IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number:	09/478,299
Applicants:	John L. Schenk
Filed:	January 5, 2000
Title:	Method of Cryopreserving Selected Sperm Cells
Confirmation No.:	1509
TC/A.U::	1654
Examiner::	Michael V. Meller
Assignee::	XY, Inc.
Attorney Docket::	22091-701
Customer No.:	33549

AFFIDAVIT UNDER 37 C.F.R. § 1.132

1. I, John L. Schenk, a resident of Fort Collins, in the County of Larimer, and State of Colorado, duly sworn and under oath, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

2. Since approximately 1981, I have been involved to varying degrees in the field of sexing spermatozoa and producing animals from such spermatozoa. At the least, this has included work with the Texas A&M University; Colorado State University; Ankony Shadow Isle; and ABS Global, Inc. In fact, I am currently employed by XY, Inc., the assignee of the above referenced patent application. I am also a co-inventor on several patents or patent applications in the field of sexing spermatozoa and producing animals from such spermatozoa, including at least: US Application 60/400,486 entitled "Sperm Cell Process Systems"; US Application 60/400,971 entitled "Low Pressure Sperm Separation System Using Heterospermic Insemination To Assess Sperm Function"; US Application 09/001,394, entitled "Sheath Fluids and Collection Systems for Sex-Specific Cytometer Sorting of Sperm"; US Application 09/015,454, entitled "System for Improving Yield of Sexed Emrbyo in Mammals"; US Application 09/448,643, entitled "Multiple Sexed Embryo Production System for Mammals"; US Application 09/478,299, entitled "Method of Cryopreserving Selected Sperm Cells"; US Application 09/582,809, entitled "Sex Specific Insemination of Mammals With Low Number of Sperm Cells"; US Application 10/081,955 entitled "Multiple Sexed Embryo Production System for Mammals Using Low Numbers of Spermatozoa"; International Application PCT/US03/24,460 entitled "Low Pressure Sperm Cell Separation System"; and International Application PCT/US00/30,146 entitled "Methods for Improving Sheath Fluids and Collection Systems for Sex-Specific Cytometer Sorting of Sperm". My duties as Reproductive Physiologist for XY, Inc. allow me to review various publications and efforts by others relevant to the field of sexing spermatozoa and producing animals from

such spermatozoa and require that I have a certain level of technical expertise in these fields.

3. By virtue of my experience and expertise in the technology of the present invention, I have knowledge and skill which is at least representative of those skilled in the art of cryopreservation of sexed spermatozoa as involved in the above referenced patent application.

4. I have reviewed the following documents:

a. the specification of U.S. Patent Application No. 09/478,299 as filed on January 5, 2000 ("the Original Application"), attached as Exhibit A;

b. the claims as filed on November 14, 2005 ("Subject Claims"), attached as Exhibit B;

c. the examiner's comments ("Subject Examination Comments"), attached as Exhibit C;

d. the article of Salisbury et al., "Physiology of Reproduction and Artificial Insemination of Cattle" 2nd Ed. San Fransciso: W.H. Freeman 442-554 (1978) ("the Salisbury reference"), attached as Exhibit D;

e. U.S. Patent No. 5,021,244 to Spaulding ("the Spaulding reference"), attached as Exhibit E; and

f. U.S. Patent No. 4,474,875 to Shrimpton ("the Shrimpton reference"), attached as Exhibit F.

5. I understand that the Subject Claims are to be reviewed by the examiner, and I understand that the bracketed or strikethrough items have been deleted and that the underlined portions are additions to the claims. Further, the Subject Examination Comments have been explained to me as having been provided by the U.S. Patent and Trademark Office, dated May 13, 2005 with respect to the above identified application.

6. Based upon knowledge and skill which is representative of those having ordinary skill in the relevant art, and after review of the Original Application, I am of the opinion that the invention as set forth in the Subject Claims is discussed and supported by the Original Application so as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Further, the Original Application and Subject Claims particularly points out and distinctly claims the subject matter of this invention. I am also of the opinion that the invention as set forth in the Subject Claims is differentiated from the Salisbury reference, the Spaulding reference, and the Shrimpton reference. The basis for these conclusions are listed below.

7. Claim 38 references the range of at least 5 million per milliliter of extender to at least about 10 million per milliliter of extender. This relates to the concentration of sperm in an extender as discussed in the specification. The values and results shown in Table 1 on page 21 and to some degree in Table 2 on page 19 highlight how the claimed ranges show significance in the data. In Table 1, it can be seen how the results increase to a more consistent value when the dilution is established at values of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender. Specifically, Table 1 shows results of the sperm motility (%) for different sperm concentrations after cooling to 5°C for either 24 hours or 48 hours. As the sperm concentration increases from 1.25 X 10^6/ml to 5 X 10^6/ml, the sperm motility increases. For example, the sperm motility at 10 X 10^6/ml is 61% for the 24 hour incubation and 42% for the 48 hour incubation. From these results, I understand that the sperm motility may level off at 5 X 10^6/ml, 10 X 10^6/ml, 15 X 10^6/ml, and perhaps even 20 X 10^6/ml dilutions. Thus, the results in Table 1 provide dilutions (e.g., at least 5 X 10^6/ml to at least 10 X 10^6/ml) that may be an appropriate basis for the minimal amount of sperm cells in extenders. While of course other base dilution values may be used, it is clear that there is support in the specification, such as shown in Table 1, for the ranges of "at least about 5 million per milliliter to at least about 10 million per milliliter of extender" as presented in claim 38. Accordingly, I conclude that support for the claimed ranges is found in the Original Application.

8. Claim 43 references the range of between about 1,000,000 to about 25,000,000 equine sperm cells. Various dosages and amounts of sperm cells are discussed in the Original Application (e.g. 50 X 10^6/ml). The volume of the straws used for insemination is also mentioned as both 0.25 ml and 0.50 ml. Applying the extension mentioned in the Original Application with these volumes of straws, it can be understood that sperm dosages of 25,000,000 (50 X 10^6/ml in a 0.50 ml straw) were used. Further, the use of 1,000,000 (1 X 10^6) sperm is explicitly mentioned.

9. As the Original Application notes, extension of sperm samples may produce a suspension of sperm, which may be then transferred into containers for freezing. The cells may be conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g. for cattle and horses) or can be readily determined. Therefore, since the Original Application mentions concentrations of sperm of 1,000,000 and 25,000,000 (50 X 10^6/ml in a 0.50 ml straw), the Original Application supports the claimed range of the concentrations of between about 1,000,000 to about 25,000,000 based on the substantial variation of doses that may be required. Accordingly, I understand that the claimed range of about 1,000,000 to about 25,000,000 of equine sperm cells is supported by the Original Application.

10. Claims 63 mentions the aspect of equilibrating for period of about 1 hour to about 18 hours. This aspect is clearly discussed in the Original Application. Further, claim 64 mentions the aspect of equilibrating for a period of not greater than 6 hours. The Original Application states, "... sperm are allowed to equilibrate for a period in the range of about

1 hour to about 18 hours, ... preferably between about 3 hours to about 6 hours...." As different times for equilibration may be desired (e.g. 1-18 hours, 3-18 hours, 3-6 hours), claims 63 and 64 covers some of the different time limitations. Further, with respect to claim 64, I understand the text of the Original Application when it states, "to about 6 hours" to describe equilibrating for a period of not greater than 6 hours. Accordingly, these claimed time limits are supported by the specification.

11. In addition, claims 63 and 64 mention equilibrating sex-selected sperm cells to a cooler, non-freezing temperature. A cooler temperature may include cooling sperm cells to a lower temperature. It may be desirable to cool the temperature of the sperm cells suspended in an extender to any temperature above freezing. As a non-limiting example, sperm can be cooled to 5 degrees Celsius. Of course, other temperatures may be used.

12. After reviewing each of the Salisbury reference, Spaulding reference, and Shrimpton reference, I do not find that any of them teach or even suggest all of the elements of independent claim 38. For example, claim 38 is distinguished from these references through the inclusion of the elements of sorting the sperm cells without the presence of protective compounds in seminal plasma and suspending at dilutions of at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender. In addition, I believe that the techniques taught in the Spaulding and Shrimpton references are not capable of being successfully reproduced.

13. Specifically, I have never personally observed a successful accomplishment of the techniques described in the Spaulding reference. I am not aware of any credible evidence suggesting a single instance in which the techniques of the Spaulding reference were successfully reproduced to achieve effective sperm cell discrimination techniques. Further, given the desirability of achieving the results claimed by the Spaulding reference, I believe that the techniques described in the Spaulding reference would be widely practiced if they could be successfully reproduced. Because I have observed that the techniques of the Spaulding reference in fact are not widely practiced, I believe that such techniques cannot be successfully reproduced to increase the probability that offspring will be of the desired sex.

14. Further, I am not aware of any credible evidence beyond that purported to be set forth in the Spaulding reference verifying the successful application to sperm cell discrimination techniques of the use of antibodies to selectively bind to sex-associated membrane proteins. Moreover, the disclosure of the Spaulding reference itself is methodologically insufficient to support a conclusion that the use of antibodies to selectively bind to sex-associated membrane proteins was successfully accomplished as a sperm cell discrimination technique. In particular, the disclosure of the Spaulding reference provides little or no data demonstrating that antibodies were selectively bound to the sex-associated membrane proteins of sperm cells to actually achieve effective separation of such sperm cells into significantly enriched populations of either X-chromosome bearing-sperm cells or Y-chromosome-bearing sperm cells. Examples of such methodological insufficiencies include, but are not limited to, the lack of an explicit statement that an actual enriched population of either X-chromosome-bearing-sperm cells

or Y-chromosome-bearing sperm cells was achieved, the lack of any data regarding what actual purities were achieved for such enriched populations, the lack of any data regarding the methodology used to establish and measure such purities, the lack of any data regarding actual artificial inseminations accomplished with such enriched populations, the lack of any data regarding the methodology used to accomplish such artificial inseminations, and the lack of any data regarding actual sex ratios achieved for the offspring of such artificial inseminations. As a result, I believe that the use of antibodies to selectively bind to sex-associated membrane proteins currently cannot be used to yield populations of sperm enriched for X-chromosome-bearing or Y-chromosome-bearing characteristics nor to increase the probability that offspring will be male or female.

15. Accordingly, it is my opinion that the techniques described in the Spaulding reference are not enabling to accomplish the techniques proposed therein, and therefore could not be successfully accomplished by a person skilled in the art to achieve such techniques.

16. I have never personally observed a successful accomplishment of the techniques described in the Shrimpton reference. Additionally, I am not aware of any credible evidence suggesting a single instance in which the techniques of the Shrimpton reference were successfully reproduced to accomplish the techniques proposed therein. Further, given the desirability of achieving the results claimed by the Shrimpton reference, I believe that the techniques described in the Shrimpton reference would be widely practiced if they could be successfully reproduced. Because I have observed that the techniques of the Shrimpton reference in fact are not widely practiced, I believe that such techniques cannot be successfully reproduced.

17. Moreover, I am not aware of any credible evidence verifying the existence of a difference in the buoyancy of X-chromosome-bearing sperm and Y-chromosome-bearing sperm, and therefore believe that techniques for the identification and discrimination of sperm cells based on such differences in buoyancy cannot be effective. With particular respect to the Shrimpton reference, I believe the evidence of such a difference presented therein is unconvincing. The only evidence of such discrimination between X-chromosome-bearing sperm and Y-chromosome-bearing sperm in the Shrimpton reference is purported to be based on sexes of fetuses from 13 inseminated cows. This evidence is not sufficiently robust to be convincing, especially in the absence of details of experimental design and any evidence of additional replication of these techniques elsewhere in the Shrimpton reference or in other publications. Further, pursuant to my review of the state of the art in this field, I have never observed even a single credible report beyond that purported to be made in the Shrimpton reference of a difference in the buoyancy of X-chromosome-bearing sperm cells and Y-chromosome-bearing sperm cells.

18. Accordingly, it is my opinion that the techniques described in the Shrimpton reference are not enabling to accomplish the techniques proposed therein, and therefore

could not be successfully accomplished by a person skilled in the art to achieve such techniques.

19. In conclusion, I disagree with the Subject Examination Comments. First, based upon the Subject Claims, I believe that the Original Application supports and shows possession of the claimed subject matter. Second, based on my review of the references and the Subject Claims, I believe that the references do not teach nor suggest the elements of claim 38. Of course, observations on other aspects may be made, however, it seems unnecessary at present.

20. One of ordinary skill in the art would agree with me in my conclusions stated above.

21. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the application or any patent issued thereon.

Dated the 20 day of January, 2006.

John L. Schenk

UNITED STATES OF AMERICA)
STATE OF COLORADO) ss.
COUNTY OF LARIMER)

SUBSCRIBED AND SWORN to before me in the County of Larimer, State of Colorado, United States of America, by John L. Schenk this 20th day of January, 2006.

WITNESS my hand and official seal pursuant to the authority vested in me as a Notary Public by the State of Colorado.

CHERYL A. SWANSON NOTARY PUBLIC STATE OF COLORADO

Cheryl A. Swanson
Notary Public
My Commission Expires: 8/21/2007

Express Mail No: EV666038705US

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Group Art Unit:	1654
Examiner:	M. Meller
Assignee:	XY, Inc.
Attorney Docket:	22091-701
Customer No.:	33549

CERTIFICATE OF EXPRESS MAILING

I, Barbara Schroeer, hereby certify to the truth of the following items:

1. I am an employee of Santangelo Law Offices, P.C., 125 South Howes, Third Floor, Fort Collins, Colorado 80521.

2. I have this day deposited the attached Affidavit under 37 C.F.R. §1.132 including Exhibits A – F with the United States Postal Service as Express Mail, postage prepaid, for mailing to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated this 20 day of January, 2006.

Barbara Schroeer

Barbara Schroeer

METHOD OF CRYOPRESERVING SELECTED SPERM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

The applications claims the benefit of U.S. Provisional Application No. 60/167,423, filed November 24, 1999.

FIELD OF THE INVENTION

The invention relates to a method for freezing sperm selected for a particular characteristic, as well as to a frozen selected sperm sample and methods of using such a sample. The invention is particularly useful for preserving sex-selected sperm.

BACKGROUND OF THE INVENTION

Over half a century ago, artificial insemination was introduced in the United States as a commercial breeding tool for a variety of mammalian species. Although artificial insemination was initially limited to regions relatively close to the site of sperm collection, advances in the cryopreservation and storage of sperm have facilitated widespread distribution and commercialization of sperm intended for artificial insemination or in vitro fertilization.

Further improvements in mammalian sperm collection, selection, cryopreservation, storage, and handling techniques have enhanced the ability of breeders to produce animals having desired traits. For example, advances in selection of mammalian sperm based on slight differences in physical characteristics has made it possible to separate sperm based on sex-type, that is, to select for cells containing either the X or Y chromosome. This technique allows the breeder to manipulate the relative percentage of X- or Y-type sperm in a sample and thereby determine offspring sex. The ability to select sperm based on sex-type or any other desirable characteristic provides an important tool for accelerating genetic progress, increasing production efficiency, and achieving greater flexibility in livestock management. Full exploitation of this tool, however, depends on the ability to freeze and store selected sperm.

A variety of methods are available for selecting cells; however, the selection and subsequent processing of sperm presents unique challenges because sperm are incapable of DNA repair and because of sperm morphology. Each sperm has an acrosome overlying the head and a tail, which are important for fertility and which are relatively susceptible to physical injury. In

addition, sperm fertility decreases with increasing time between collection and use. As most of the available selection methods involve physical stresses and take time, selected sperm are typically somewhat compromised compared to non-selected cells. Fertility may be further reduced if the selection technique involves significant dilution. It has been suggested that this "dilution effect" may be due to the loss of protective components in seminal plasma.

Flow cytometry is a particularly efficient selection method that has been employed for sorting sperm by sex-type. However, sorted sperm are subject to stresses beyond those normally encountered in standard artificial insemination or in vitro fertilization protocols. In particular, flow cytometry is time consuming, and, because of the physical constraints of flow cytometers, sperm must be diluted for sorting to levels that are not optimal for storage (usually to on the order of 10^5-10^6/ml). Furthermore, sorted sperm intended for artificial insemination must be concentrated so that conventional packaging and delivery equipment can be used. The need for a concentration step thus exposes already somewhat compromised sperm to additional physical stresses.

The freezing of sperm also invariably reduces fertility, motility, and/or viability, and, although techniques for freezing unselected sperm are well known, no technique for cryopreservation of selected sperm has been described.

SUMMARY OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. The method is particularly useful for cryopreserving sperm selected by a method that results in dilution of the sperm, since the method provides for the isolation of sperm from a selected sperm sample, followed by addition of a final extender to the isolated sperm to produce a suspension having a desired concentration of sperm. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred.

The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or

bison sperm. Also within the scope of the invention is a container including a frozen sperm sample according to the invention.

The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows cryopreservation of sperm that have been selected for a particular characteristic, facilitating storage and/or shipment of selected sperm samples to sites distant from the collection site. Thawing yields viable sperm that can be used in procedures such as artificial insemination ("AI") and in vitro fertilization ("IVF"). This result was surprising because of the well-documented fragility of sperm. Prior researchers had demonstrated that the stresses associated with various selection methods or with cryopreservation resulted in significant losses in fertility and/or viability. The present inventors have demonstrated, for the first time, that pregnancies can be achieved with sperm that have been selected and then frozen.

The invention represents an important advance in livestock management, where selection of sperm for use in such procedures can be used to increase the production of offspring having desirable traits. For example, selection to obtain sperm carrying either the X or the Y chromosome allows control over offspring sex, which is advantageous for producers of animals such as dairy or beef cattle. Sex selection also finds application in breeding valuable (e.g., show or race horses) or endangered animals. The ability to freeze selected sperm, which the invention provides, will enable widespread use of such selection methods to, e.g., increase livestock production efficiency as well as quality.

Definitions

The term "acrosome" or "acrosomal cap" refers to the cap that covers the anterior half of the head of sperm and that contains enzymes necessary for ovum penetration.

The term "sex-type" refers to the type of sex chromosome present in the sperm (i.e., the X or Y chromosome).

The term "capacitation" refers to the specific changes a sperm undergoes to develop the capacity to fertilize ova, such as enzymic changes on the surface of the acrosome that lead to release of acrosomal enzymes that facilitate penetration of the sperm into the ovum.

As used with reference to sperm, the term "cryoprotectant" refers to a molecule that protects sperm during a freeze-thaw cycle, promoting survival and retention of fertilizing capacity.

The term "dilution effect" refers to the rapid decline in motility and/or viability of sperm when highly diluted.

As used herein, the term "selection" refers to a method whereby a sample is subdivided based on presence or absence of a specific characteristic (unless context dictates otherwise). Thus, a "selected sperm sample" is a sample obtained by subjecting a source sample to selection for the specific characteristic. A selected sperm sample is therefore enriched, relative to the source sample, in sperm having the specific characteristic.

The term "sorting" is used herein to describe a selection method carried out using a fluorescence-activated cell sorter (FACS).

The term "extender" refers to any medium that tends to preserve sperm viability. The term "extension" refers to the dilution of sperm with extender.

The term "initial extender" refers to a medium used to extend sperm prior to the isolation step of the method of this invention.

The term "final extender" refers to a medium used to extend sperm prior to the freezing step of the method of this invention.

An "organic substance" in an extender described herein is any organic substance that tends to reduce cold shock and preserve fertility of sperm.

An "energy source" in an extender described herein is any substance or substrate that sperm can utilize for cell maintenance and/or motility.

The term "osmolality," as used herein, is a measure of the osmotic pressure of dissolved solute particles in a an aqueous solution (e.g., an extender). The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water.

Cryopreservation Method

The invention provides a method of cryopreserving selected sperm includes the following steps:

(1) obtaining a selected sperm sample;

(2) cooling the selected sperm sample;

(3) isolating sperm from the selected sperm sample;

(4) adding final extender to the isolated sperm to produce a suspension of sperm; and

(5) freezing the suspension of sperm.

Obtaining a Selected Sperm Sample

The first step in the cryopreservation method of the invention encompasses obtaining a previously selected sperm sample, as well as subjecting a source sample to any suitable selection method. Sperm from any species can be selected and frozen according to the method of the invention. The method can be carried out with sperm from domesticated animals, especially livestock, as well as with sperm from wild animals (e.g., endangered species). Preferably, the selected sperm sample contains mammalian sperm. Human sperm, bovine, equine, porcine, ovine, elk, and bison sperm are particularly preferred.

Generally, the selected sperm sample contains normal, viable sperm. To this end, the ejaculate from which the sperm are obtained typically has at least about 50%, and preferably at least about 75% morphologically normal sperm. In these embodiments, generally at least about 40%, and preferably at least about 60% of the sperm in the ejaculate exhibit progressive motility.

A wide variety of methods for selecting cells from a mixed populations are available, including, for example, selection based on binding of cells or cell components with antibodies, antibody fragments, or other binding partners and differential staining.

The invention is exemplified herein with selection based on sex-type, and sex-selected sperm for use in the invention can be obtained using any selection strategy that takes advantage of slight differences in characteristics between X- and Y-type sperm. Exemplary sex-selection methods include magnetic techniques (see, e.g., U.S. Patent No. 4,276,139), columnar techniques (see, e.g., U.S. Patent No. 5,514,537) gravimetric techniques (see, e.g., U.S. Patent No. 3,894,529, reissue Patent No. 32350, U.S. Patent Nos. 4,092,229, 4,067,965, and 4,155,831). Sex-selection based on differences in electrical properties is disclosed in U.S. Patent

No. 4,083,957, and techniques that select based on differences in electrical and gravimetric properties are discussed in U.S. Patent Nos. 4,225,405, 4,698,142, and 4,749,458. U.S. Patent Nos. 4,009,260 and 4,339,434 describe selection based on differences in motility. Biochemical techniques relying on antibodies are disclosed in U.S. Patent Nos. 4,511,661, 4,999,283, 3,687,803, 4,191,749, 4,448,767, whereas U.S. Patent Nos. 5,021,244, 5,346,990, 5,439,362, and 5,660,997 describe selection based on differences in membrane proteins.

Flow cytometry is a preferred method for separating cells from mixed populations based on differential staining with fluorescent dyes or binding to fluorescently labeled molecules, such as antibodies or nucleic acids. In fluorescence activated cell sorting ("FACS"), cells are "sorted" into different populations based on the fluorescence intensity upon irradiation. FACS can be used for sex-selection of sperm because the X chromosome contains slightly more DNA than the Y chromosome. When sperm are stained with a fluorescent DNA-binding dye, X-chromosome bearing sperm absorb more dye than Y-chromosome bearing sperm and the two populations can therefore can be separated by FACS. This strategy was discussed in U.S. Patent No. 4,362,246 and significantly expanded upon in U.S. Patent No. 5,135,759 (issued to Johnson). Separation has been enhanced through the use of high-speed flow cytometers, such as the MoFlo® flow cytometer produced by Cytomation, Inc. (Ft. Collins, CO) and described in U.S. Patent Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796, as well as in PCT Publication No. WO 96/12171.

The selection method used to obtain the selected sperm sample is preferably one that preserves sperm viability. Because of the relative fragility of sperm, normal flow cytometry techniques should generally be modified for sorting sperm. More specifically, the flow cytometry entails staining, dilution, and interrogation of cells with light. All of these steps represent stresses that can reduce sperm viability. The sensitivity of sperm to these stresses can vary between species and even between individuals within species. Such sensitivities have either been documented or can readily be determined by empirical studies, such as those described in Examples 1-5.

Modifications that enhance viability are described the patent publications discussed above. For instance, procedures that provide improved sheath and collector systems for sorting sperm are disclosed in PCT Publication No. WO 99/33956 (Application No. PCT/US98/27909). Further, Examples 1-7 below describe exemplary procedures for staining and sorting sperm.

Example 3 describes a study of the effects of laser intensity and dye concentration of post-thaw motility of sorted frozen sperm. This study indicates that the use of lower laser intensities during sorting can increase post-thaw motility.

The selected sperm sample can contain a variety of components besides sperm and will often contain components added to protect the sperm during the selection process. In the case of FACS, the selected sperm sample can contain component(s) of the solutions used for staining and sorting (e.g., the sheath fluid and the catch buffer).

In addition, the selected sperm sample typically contains an extender or extender fraction. For example, "two-step" extenders including an "A fraction" lacking glycerol and a "B fraction" containing glycerol are well known. The A fraction is added to sperm first, followed by addition of an equal volume of the B fraction. For this step, the B fraction is often divided into at least two aliquots and added sequentially; e.g., the second B fraction aliquot is added 15 minutes after the first.

If no extender components are present, an extender or extender fraction is typically added to the selected sperm sample before the sperm are isolated from the sample. If only some extender components are present, additional components can optionally be added so that selected sperm sample includes a complete extender or an extender fraction before the isolation step. In exemplary embodiments, bovine sperm are flow-sorted so as to produce a selected sperm sample including the A fraction of an extender (see Examples 2, 3, and 4). If desired, the B fraction can then be added to the selected sperm sample before the isolation step (see Example 5). The pre-isolation step extender (or extender fraction) is termed "the initial extender" to distinguish it from the "final extender" employed for the extension of isolated sperm before freezing. If the selected sperm sample was selected using FACS, the initial extender can be matched to the sheath fluid employed for sorting. Exemplary matched sheath fluids and extenders are described in detail in Example 4.

An extender suitable for use in the selected sperm sample includes a physiologically acceptable carrier. The physiologically acceptable carrier is typically aqueous, and, in preferred embodiments, includes deionized water. Suitable extenders commonly comprise one or more of the following additional components: a cryoprotectant, a component that maintains osmolality and buffers pH, an organic substance that prevents cold shock and preserves fertility of sperm, a detergent that acts synergistically with certain organic substances to enhance preservation of

sperm, an energy source that can be readily utilized by sperm, an antioxidant, which protects sperm from cold shock, a substance that facilitates sperm capacitation, and one or more antibiotics.

Although cryoprotectants useful in the invention are not limited to those acting by a particular mechanism, most conventional cryoprotectants act, at least in part, by reducing intracellular dehydration. Specifically, freezing is accompanied by an increase in solute concentration in the medium surrounding sperm. This increase in solute concentration draws water out of the cells, which increases intracellular electrolyte concentration. Exemplary cryoprotectants include glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and the like. The cryoprotectant suitable for use in a given extender can vary, depending on the species from which sperm are derived. For example, glycerol is suitable for use in cryopreservation of human and bovine sperm, but is generally not used for cryopreservation of porcine or rabbit sperm. Such preferences are well known for many commercially valuable sperm and can readily be determined empirically for other types of sperm.

The extender useful in the invention optionally includes one or more components that help maintain osmolality and provide buffering capacity. In preferred embodiments of the invention, the osmolality of the extender approximates that of physiological fluids. More preferably, the osmolality of the extender is in the range of about 280 mOsm to about 320 mOsm. The pH is also preferably within a physiologically acceptable range, more preferably in the range of about 6.5 to about 7.5.

Substances helpful in maintaining omolality and pH within these ranges are well known in the art and can be added to the extender as a solid or already in solution. A buffer containing a salt, a carbohydrate, or a combination thereof can be employed for this purpose. Specific examples include sodium citrate, Tris[hydroxymethyl]aminomethane, and TES (N-Tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid), and monosodium glutamate buffers; milk; HEPES-buffered medium; and any combination thereof. The component employed to help maintain osmolality and provide buffering capacity in a particular application can vary depending on the other components of the extender and, in some cases, on the species from which the sperm are derived. The selection of such a component for use in the present invention is, however, within the level of skill in the art.

One or more organic substances that protect sperm against cold shock and help preserve fertilizing capacity can also be included in the extender. Such substances are well known and are sometimes described as "nonpenetrating cryoprotectants." One skilled in the art can readily determine an organic substance suitable for a particular application of the cryopreservation method described herein. For example, organic substances containing protective constituents (e.g., lipoproteins, phospholipids, lecithin) that are believed to reduce the impact of cold shock and the dilution effect can be included in the extender. Suitable organic substances include disaccharides, trisaccharides, and any combination thereof. Exemplary organic substances include egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, cholesterol, and any combination thereof.

The extender can also include a detergent. Alkyl ionic detergents, such as sodium dodecyl sulfate (SDS), have been reported to act synergistically with egg yolk to enhance protection against cold shock. Other detergents useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular detergent for a specific application is within the level of skill in the art in light of the guidance provided herein. See, e.g., Example 5.

Preferably, the extender includes an energy source that is readily utilized by sperm. In the absence of an energy source, sperm may oxidize intracellular phospholipids and other cellular components. Thus, the inclusion of an energy source in the extender protects intracellular reserves and cellular components. As is well known in the art, sugars, particularly monosaccharides, provide a convenient energy source, although any conventional energy source can be employed in the extender. Exemplary monosaccharides useful in the extender include glucose, fructose, and/or mannose.

One or more antioxidants can optionally be included in the extender to provide additional protection against cold shock. Exemplary antioxidants include butylated hydroxytoluene (BHT), its derivatives, and the like. However, other antioxidants useful in the cryopreservation of cells can also be employed in the extender, and the selection of a particular antioxidant for a specific application is within the level of skill in the art in light of the guidance provided herein.

The extender can also contain a substance that facilitates sperm capacitation. A variety of capacitation facilitators are known in the art and any can be employed in the extender. Examples

include enzymes such as alpha amylase, beta amylase, beta glucuronidase, which can be used in combination, if desired.

Finally, the extender preferably includes an antibiotic, since substantial bacterial growth can threaten sperm viability and increase the risk of infection of the host in artificial insemination or in vitro fertilization procedures. Any of a variety of antibiotics useful in the cryopreservation of cells can also be employed in the extender. The selection of a suitable antibiotic depends on the species from which the sperm was obtained, the procedures involved in obtaining and handling the sperm sample, and the specific microorganism(s) to be targeted. Exemplary antibiotics include tylosin, gentamicin, lincomycin, spectinomycin, linco-spectin (a combination of lincomycin and spectinomycin), penicillin, streptomycin, and ticarcillin, which can be used alone or in combination. However, one skilled in the art can readily determine other antibiotics suitable for use in the extender.

Exemplary extenders are discussed in greater detail below and in the examples.

The sperm concentration is typically lower in the selected sperm sample than in the source sample, and, as indicated above, when FACS is employed, the dilution is significant. A typical sort based on sex-type can produce a sample containing sperm at 6×10^5 cells/ml catch buffer. As such a low concentration is not optimal for storage (at least for most species tested), the cryopreservation method of the invention generally concentrates the selected sperm sample.

Cooling the Selected Sperm Sample

The second step in the cryopreservation method entails cooling the selected sperm sample, typically, by reducing the temperature at a controlled rate. Cooling too rapidly can cause cold shock, which can result in a loss of membrane integrity and cell function. The severity of the effects of cold shock vary from species to species and depend on factors such as the rate of cooling and the temperature range. Under suitable controlled cooling conditions, the sperm are able to adapt to thermal effects. Example 2, among others, describes conditions for cooling bovine sperm, and determining suitable conditions for cooling sperm of other species is within the level of skill in the art.

In a preferred embodiment of the invention, the selected sperm sample is cooled typically from about 22°Celsius, to about 5°Celsius, and cooling is generally carried out over a period of about 60 minutes to about 24 hours, preferably over a period of about 90 minutes to about 240

minutes, and most preferably over a period of about 90 minutes to about 120 minutes. Cooling can be accomplished by any convenient method, including simply placing the selected sperm sample in a 5°Celsius environment.

Isolation of Sperm Cells from the Selected Sperm Sample

After initial extension of the selected sperm sample, sperm are isolated from the sample using any sufficiently gentle isolation method that provides at least about 50% recovery of sperm, more preferably about 75% to about 90% recovery of sperm, and most preferably about 80% to about 90% recovery of sperm. During the isolation step, the cooled sperm should generally be kept cold, i.e., between about 1 and about 8°Celsius, and preferably close to 4 or 5°Celsius.

Any of a variety of methods suitable for recovering cells from a suspension can be used to isolate the sperm, including for example, filtration, sedimentation, and centrifugation. In an exemplary, preferred embodiment, the selected sperm sample is aliquoted into 50 ml tubes at volumes not exceeding about 27 ml, and preferably between about 20 to about 27 ml. Centrifugation is carried out at about 4°Celsius, at about 850 x g, for about 20 minutes. Preferably, the centrifugation step provides at least about 50% to about 90% recovery of sperm, more preferably about 60% to about 90% recovery of sperm, and most preferably about 70% to about 90% recovery of sperm. After isolation, the supernatant is removed and the pellet is suspended by vortexing gently or repeated aspiration at 4°Celsius. The sperm concentration is then typically determined (e.g., using a hemacytometer).

Final Extension of Isolated Sperm Cells

After isolation, the sperm are pooled, if desired, and extended with final extender to an appropriate concentration for freezing. The concentration of sperm after the final extension and prior to freezing is preferably in the range of about 1 x 10^6/ml to about 300 x 10^6/ml, more preferably about 10 x 10^6/ml to about 50 x 10^6/ml, and most preferably about 10 x 10^6/ml to about 20 x 10^6/ml.

The description of the initial extender above also applies to the final extender, which can be the same as or diffcrent from the initial extender. In particular embodiments, the composition

of the sperm sample extended with the final extender is substantially similar to (if not the same as) the composition of the sperm sample after addition of the initial extender.

In a preferred embodiment of the invention, an egg yolk-Tris extender is used. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); citric acid and Tris[hydroxymethyl]aminomethane (buffer); egg yolk (organic substance); fructose (energy source); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk-Tris Extender

Glycerol:	4-8% vol/vol
Citric Acid:	55-75 mM
Tris [hydroxymethyl]aminomethane:	190-210 mM
Egg yolk:	5-25% vol/vol
Fructose:	45-65 mM
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/ml*

*100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

In a variation of this embodiment particularly suitable for freezing bovine sperm, the concentrations of these components after addition of the final extender to the isolated sperm are about 6% (vol/vol) glycerol, about 65 mM citric acid, about 200 mM Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 56 mM fructose, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 150/300 µg/ml linco-spectin (i.e., 150 µg/ml lincomycin and 300 µg/ml spectinomycin), in deionized water.

In an alternative embodiment, an egg yolk-citrate extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); sodium citrate (buffer); egg yolk (organic substance); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk-Citrate Extender

Glycerol:	4-8% vol/vol
Sodium Citrate :	60-80 mM
Egg yolk:	5-25% vol/vol
Tylosin:	25-100 µg/ml
Gentamicin:	200-300 µg/ml
Linco-spectin:	100-400 µg/mL*

*100-400 µg/ml lincomycin and 100-400 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 7% (vol/vol) glycerol, about 72 mM sodium citrate, about 20% (vol/vol) egg yolk, about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

In another alternative embodiment, an egg yolk-TES-Tris ("EY TEST") extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); egg yolk and heated milk, e.g., homogenized milk containing 1.25% fructose with 10% glycerol (organic substances); tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Egg Yolk TES-Tris Extender

Glycerol:	3-7% vol/vol
Tris [hydroxymethy-methyl]-2-aminoethanesulfonic acid:	140-170 mM
Tris [hydroxymethyl]aminomethane:	60-80 mM
Egg yolk:	5-25% vol/vol
Fructose:	5-12 mM
Tylosin:	50-150 µg/ml
Gentamicin:	400-600 µg/ml
Linco-spectin:	200-700 µg/mL*

*200-700 µg/ml lincomycin and 200-700 µg/ml spectinomycin

Exemplary, preferred concentrations for freezing bovine sperm are about 5% (vol/vol) glycerol, about 158mM Tris[hydroxymethy-methyl]-2-aminoethanesulfonic acid, about 72 mM Tris[hydroxymethyl]aminomethane, about 20% (vol/vol) egg yolk, about 8mM fructose, about 100 µg/ml tylosin, about 500 µg/ml gentamicin, and about 300/600 µg/ml linco-spectin.

In yet another alternative embodiment of the invention, a Milk extender is employed. After the addition of the extender, the sperm suspension comprises glycerol (cryoprotectant); heated homogenized milk (organic substance); fructose (energy source); and tylosin, gentamicin, and linco-spectin (antibiotics). The typical approximate concentrations of these components after addition of the final extender to the isolated sperm are:

Components of Milk Extender

Homogenized Milk	90% (vol/vol)
Glycerol:	3-7% (vol/vol)
Fructose:	1.25 % (wt/vol)
Tylosin:	50 µg/ml
Gentamicin:	250 µg/ml
Linco-spectin:	250/300 µg/ml

250-300 µg/ml lincomycin and 250-300 µg/ml spectinomycin

Exemplary preferred concentrations for freezing bovine sperm are about 90% milk, about 10% (vol/vol) glycerol, about 1.25% fructose (wt/vol?), about 50 µg/ml tylosin, about 250 µg/ml gentamicin, and about 250/300 µg/ml linco-spectin.

Other extenders standardly used to freeze sperm can also be employed as the final extender in freezing selected sperm. A variety of extenders optimized for use in freezing sperm from various species have been described, and many are commercially available. Freezing extenders for equine sperm typically consist of milk, egg yolk, various sugars, electrolytes and a cryoprotectant. Exemplary freezing extenders are described by Squires, E.L., et al., Cooled and Frozen Stallion Semen Animal Reprod. and Biotechnology Laboratory, Bulletin No. 69, Chapter 8, "Seminal Extenders" pp. 49-51 (July, 1999).

Equilibration and Freezing of Sperm

Extension of the sperm sample produces a suspension of sperm, which is then transferred into containers for freezing. If the sperm are intended for use in fertilization, the cells are conveniently aliquoted into individual doses sufficient to achieve fertilization. The required dose can vary substantially from one species to the next and is either well-known (e.g., for cattle and horses) or can readily be determined. In the case of sex-selected bovine sperm, convenient doses range from about 1.0×10^6 sperm to about 3.0×10^6 sperm.

Any suitable container can be employed for freezing, including, for example, an ampule, a vial, and a straw. Sperm intended for AI are typically frozen in straws (e.g., 0.25 ml or 0.50 ml

straws) designed for use with an insemination gun. Preferably, a bolus of extender is drawn into the straw, followed, in sequence, by air, sperm, air, and extender, so that the sperm are flanked on either side by an air space, which separates the sperm from a bolus of extender at either end of the straw.

Prior to freezing, the sperm are generally allowed to equilibrate at about 5°C. Preferably, the sperm are allowed to equilibrate for a period in the range of about 1 hour to about 18 hours, more preferably between about 3 hours and about 18 hours, and most preferably between about 3 hours and about 6 hours (see Example 2).

Following equilibration, any standard freezing method can be employed, provided the freezing rate is not too rapid (i.e., not in excess of about 0.5°C/minute). Preferably, the freezing rate is about 0.5°C/minute. In an exemplary, preferred embodiment, the sperm are placed in static liquid nitrogen vapor, and freezing is carried out in three distinct stages over a period of about 10 minutes. In the first stage of freezing, the sperm are cooled from about 5°C to about $^{-}$15°C at a rate of about 40°C/minute to about 65°C/minute. In the second stage of freezing, the sperm are cooled from about $^{-}$15°C to about $^{-}$60°C at a rate of about 25°C/minute to about 35°C/minute. In the third stage, the sperm are plunged into liquid nitrogen at about $^{-}$100°C.

Selected Sperm Samples

In addition to a freezing method, the invention provides a frozen sperm sample including sperm selected from a source sample for a particular characteristic. The sperm can be from any species, including any of those discussed above with reference to the freezing method. The invention encompasses frozen sperm selected for any characteristic by any suitable method, such as those described above. Preferred embodiments include frozen sex-selected human, bovine, equine, porcine, ovine, elk, or bison sperm. Sex-selection is preferably carried out using flow cytometry as described generally above.

Also within the scope of the invention is a container containing a frozen sperm sample according to the invention. The container can be formed from any material that does not react with the frozen sperm sample and can have any shape or other feature that facilitates use of the sample for the intended application. For samples intended for use in AI, for example, the container is conveniently a straw (e.g., 0.25 ml or 0.5 ml straw) designed for use with an insemination gun. The container is sealed in any manner suitable for preserving the sample at

the intended storage temperature, which is typically below -80°Celsius. 0.25 ml straws can be sealed, for instance, with PVC powder, ultrasonically, or with a cotton-polyvinyl plug and/or a stainless steel ball (BB).

As the frozen sperm sample of the invention is typically thawed before use, the invention also provides a thawed, previously frozen, selected sperm sample and a container including such a thawed sample.

Methods of Using the Selected Sperm Sample

The frozen selected sperm sample of the invention is suitable for use in any method in which sperm are used. The sample can be thawed and used in any conventional fertilization method, such as artificial insemination or in vitro fertilization. Thawing is carried out in the same manner as for frozen, non-selected sperm. Briefly, the straw containing the frozen sperm is submerged in a water bath maintained at a temperature of about 35°C to about 37°C for a period of about 20 to about 30 seconds. After thawing, semen deposition (e.g., insemination) is carried out according to standard procedures, taking care to protect the sperm from environmental fluctuations.

EXAMPLES

Example 1

Effects Of Dilution On Sperm

Objective: to determine the effect of sperm concentration on sperm motility for non-frozen, non-sorted, but highly diluted sperm.

A. *Effects of Dilution on Non-washed Sperm*

1. *Collection of Source Sample*. Sperm were collected from bulls on a routine collection schedule using an artificial vagina as described in Schenk J., Proc 17th NAAB, p.48-58 (1998), and Saacke RG, Proc NAAB Tech Conf AI Reprod. 41:22-27 (1972). All ejaculates used contained greater than 50% progressively motile and greater than 75% morphologically normal sperm. Antibiotics were added to the raw ejaculate as described by Shin S., Proc NAAB Tech Conf AI Reprod. 11:33-38 (1986) within 15 minutes of collection, and the concentration of sperm was determined using a spectrophotometer.

2. *Methods*. Sperm from 4 bulls were diluted to 1.25, 2.5, 5, 10, 15, and 20 × 10^6/ml using an egg yolk-citrate extender (EYC) prepared with 20% egg yolk (vol/vol) in 72 mM sodium citrate, 50 µg/ml tylosin, 250 µg/ml gentamicin, and 250/300 µg/ml linco-spectin. Each sample was prepared in duplicate (2 tubes/dilution/bull) and comprised 8 ml total volume per tube. All samples were incubated for 60 minutes at 22°C, after which they were centrifuged using a swinging bucket centrifuge (Eppendorf, Model # 5810R) at 600 × g for 10 minutes to concentrate the sperm. After centrifugation, the supernatant from one set of the duplicate tubes was not removed; the sperm were resuspended in the same medium and at the original concentration by repeated gentle aspiration using a 5-ml serological pipette. (The second set of the duplicate tubes were used in Example 1B.) Sperm samples were then cooled to 5°C at 0.2°C/min over 90 minutes. These sperm were termed "non-washed sperm." All samples were incubated at 5°C for 24 or 48 h post-collection.

3. *Evaluation of Motility*. After incubation, the samples were warmed to 37°C using a dry block incubator for 10 minutes prior to determination of motility. For this experiment, a

single, blind estimate of the percentage of progressively motile sperm was determined for each sample. Progressive sperm motility was determined subjectively for each subclass by a single observer (x200, phase-contrast microscopy); another person prepared the microscope slides in a randomized manner so the observer was unaware of treatments.

4. *Statistical Analysis*. Data were analyzed by analysis of variance (SAS Institute, Cary, North Carolina) with factors bulls and initial dilution concentration. Separate analyses were done for each incubation time. Dilution trends were tested using (log) linear contrasts.

5. *Results*. Data for non-washed sperm (Table 1) revealed (log) linear relationships ($P<0.01$) for both incubation times. Percentages of motile sperm increased as sperm concentration increased from 1.25×10^6/ml to 10×10^6/ml, but there was little difference thereafter. The cubic term was significant ($P<0.05$) for 24-h and marginally significant ($P<0.1$) for 48-h incubations. There was a bull effect ($P<0.01$) at both times.

Table 1. Effects of cooling on non-washed sperm motility (%) after cooling to 5°C.

Dilution (10^6/ml)	Incubation at 5°C	
	24 h[a]	48 h[b]
1.25	18[c]	0[c]
2.5	38[c,d]	6[c,d]
5.0	56[d]	31[d,e]
10.0	61[d]	42[e]
15.0	55[d]	44[e]
20.0	58[d]	41[e]
S.E.[f]	5.6	6.4

[a] (log) linear ($P<0.01$) and cubic effects ($P<0.05$).

[b] (log) linear ($P<0.01$) and cubic effects ($P<0.1$).

[c,d,e] Means within columns without common superscripts differ ($P<0.05$).

[f] $\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$
(SAS Institute, Cary, NC, USA)

B. *Effects of Dilution on Washed Sperm*

1. *Collection of Source Sample*. The second set of the duplicate tubes containing samples prepared in Example 1A were used in this experiment.

2. *Methods*. The sperm were diluted, incubated and concentrated by centrifugation as in Example 1A. Following centrifugation, 7.1 ml of the supernatant was aspirated from each tube, removing most of the seminal plasma and leaving the sperm in a 900-µl pellet. The sperm were diluted with EYC (see Example 1A) to make 10×10^6/ml or 20×10^6/ml sperm suspensions. The samples were then cooled to 5°C over 90 minutes as in Example 1A.

3. *Evaluation of Motility*. The samples were warmed and evaluated for progressive motility as in Example 1A.

4. *Statistical Analysis*. Data were analyzed as in Example 1A. In addition, data in Example 1B were analyzed for incubation concentration at 5°C.

5. *Results*. Data for washed sperm (Table 2) revealed no significant treatment effects when sperm were evaluated after 24 h. However, after storage for 48 h at 5°C, there were bull, initial dilution, incubation concentration and bull by incubation effects ($P<0.05$). More sperm remained motile when held at 20×10^6 /ml than at 10×10^6 /ml (31% vs. 20%; $P<0.05$). Initial dilutions of 1.25, 2.5, and 5×10^6 sperm/ml resulted in lower progressive motility than 10×10^6 sperm/ml ($P<0.05$), with respective main effect means of 19, 20, 27, and 37% motile sperm.

Table 2. Cumulative effects of washing, dilution, concentration and cooling on progressive sperm motility (%)

	Storage at 5°C – Sperm Concentration and Duration			
	24 h		48 h[a]	
Sperm conc (10^6/ml) during 1 h preincubation at 37°C	20×10^6/ml	10×10^6/ml	20×10^6/ml	10×10^6/ml[b]
1.25	45	49	24	15
2.5	51	40	29	11
5.0	54	54	32	21
10.0	51	50	40	34
15.0	60		41	
20.0	55		40	

[a] Concentration to 20×10^6 sperm/ml was superior ($P<0.05$) to 10×10^6 sperm/ml after 48 h storage.
Also, initial dilution to 10×10^6 was superior to lower dilutions ($P<0.05$).
Pooled standard errors ($\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$ were 4.0 for 24 h, and 2.8 for 48 h incubations.

[b] Significant (log) linear trend ($P<0.06$).

C. *Conclusion*

High sperm dilution and cooling resulted in a substantial reduction in the percentage of motile sperm, regardless of the presence or removal of seminal plasma. However, this dilution

effect was greatly attenuated by concentrating the diluted sperm to 10×10^6/ml and even more, to 20×10^6/ml before storage at 5°C. Sperm from some bulls tolerated dilution better than sperm from other bulls; however, the bull differences found are typical. Extremely dilute sperm might be compromised during sorting, in part, by removal of protective compounds in seminal plasma.

Example 2

Effects of Equilibration Time Before Freezing Sorted Sperm

Objective: to evaluate the effect of equilibration times (3, 6 and 18 h, 5°C) before freezing on flow-sorted sperm.

The following experiment was replicated in its entirety using the same bulls:

1. *Collection of Source Sample*. Sperm of 4 bulls were collected and prepared as described in Example 1A.

2. *Methods*.

a) *Staining and Preparation for Sort.*

i) Preparation of Stain Stock Solution: a stock solution of 8.89 mM Hoechst 33342 (bis-Benzimide H-33342; #190305, ICN Biomedicals Inc., Aurora, OH) was prepared in deionized water.

ii) Sperm Stain Procedure: sperm were diluted in a modified TALP buffer (Table 3) to 400×10^6 sperm/ml. Following dilution, Hoechst 33342 dye was added to the sperm suspensions at a concentration of 224 μM. After the stain was added to the sperm suspensions, the samples were incubated for 60 minutes at 34°C. Following incubation, sperm were diluted to 100×10^6/ml with TALP containing 2.67% clarified egg yolk and 0.002% food coloring dye (FD&C #40) which quenches the fluorescence of Hoechst 33342 in sperm with compromised cell membranes, allowing them to be gated out during the sorting process. Just prior to flow sorting, samples were filtered at unit gravity through a 40-μm nylon mesh filter to remove any debris and/or clumped sperm.

b) *Sorting*. A two-line argon laser operating at 351 and 364 nm and 150 mW was used to excite the Hoechst 33342 dye. The flow cytometer/cell sorter used was an SX MoFlo® (Cytomation, Inc., Fort Collins, CO, USA) operating at 50 psi. A Tris-based sheath fluid was used, consisting of Tris (hydroxymethyl) aminomethane (Tris; 197.0 mM; #T-1503, Sigma Chemical Co., St. Louis, MO, USA), citric acid monohydrate (55.4 mM; #C-7129, Sigma Chemical Co., St. Louis, MO, USA) and fructose (47.5 mM; #F-0127, Sigma Chemical Co., St. Louis, MO, USA). Baseline antibiotics were also added to the Tris-based sheath fluid consisting of 0.58 g/L of penicillin and 0.05 g/L of streptomycin sulfate.

The sperm were sorted by a process referred to as "bulk sorting" which permits rapid accumulation of large numbers of sperm so that large-scale examples can be done within a reasonable time. The sperm pass through the flow cytometer under the standard operating conditions with the exception that all droplets containing viable sperm were collected into a single tube rather than being sorted into 2 tubes based upon specific characteristics (e.g., sorting by sex-type). Sperm were sorted on the basis of viability; hence, sperm that have compromised plasma membranes were excluded during bulk sorting.

Stained sperm were maintained at 22 ± 1°C during sorting. Bulk sorted sperm were collected in 50-ml plastic tubes containing 2 ml of 20% egg yolk-Tris extender prepared with 20% egg yolk (vol/vol) in 200 mM Tris, 65 mM citric acid, 56 mM fructose, 50 µg/ml tylosin, 250 µg/ml gentamicin, and 150/300 µg/ml linco-spectin in deionized water. The egg yolk-Tris extender was termed "Tris-A fraction" to denote the lack of glycerol at this point in the procedure. Sperm were collected in tubes to contain 12 ml and approximately 6×10^6 sperm. The sperm were subsequently incubated at 22°C for 1 to 3 h to simulate conditions of a sort based on sex-type.

c) *Preparation for Freezing.* Following incubation, the sorted sperm were cooled to 5°C over the period of 70 minutes. After cooling, the contents of the two tubes were pooled and transferred to a refrigerated, swinging bucket centrifuge set at 5°C and

centrifuged at 850 × g for 20 minutes. After removing the supernatant, processing continued at 5°C by adding about 150 µl of Tris-A fraction extender to about 150-µl of sperm pellet to bring the sperm concentration to approximately 40×10^6/ml. The sperm of individual bulls were pooled and diluted immediately with an equal volume of egg yolk-Tris extender containing 12% (v/v) glycerol ("Tris-B fraction"). The Tris-B fraction was added to the sperm suspension as 2 equal volumes at 15-minute intervals to adjust the final sperm concentration to 20×10^6/ml. The final glycerol concentration of the complete egg yolk-Tris extender containing the sperm was 6% (v/v).

d) *Equilibration and Freezing*. Extended sperm were then packaged into 0.25-ml polyvinylchloride straws to be frozen by routine procedures on racks in static liquid nitrogen vapor. Two straws from each of 4 bulls were frozen after 3, 6 and 18 h of total equilibration time at 5°C.

3. *Evaluation of Post-Thaw Motility*. Straws were thawed in a 37°C water bath for 30 sec. Blind estimates of progressive motility were made after incubating samples at 37°C for 0, 1 and 2 h post-thawing. Each of two observers estimated progressive sperm motility from each of two straws of semen. These four blind estimates for each experimental unit represent subsampling.

4. *Statistical Analysis*. Statistically, the subsamples were analyzed as a subplot to the main plot least-squares ANOVAs to analyze effects of any observer and observer x treatment interaction. N refers to the number of experimental units, not subsamples; standard errors were calculated on the basis of means of the 4 subsamples from error mean squares of ANOVAs and the numbers of experimental units; least-squares means are presented.

Treatment effects were evaluated via separate ANOVAs for each incubation time. The model included bulls as a random effect and equilibration time and observer as fixed effects; the subplot consisted of the observer term and related interactions.

5. *Results*. The 3- or 6-h equilibration times were superior to 18-h (Table 4), based on the percentage of progressively motile sperm, for 0 and 1 h ($P<0.01$) but not 2 h of post-thaw incubation. Effects of bull were evident at 1 and 2 h incubation times ($P<0.05$), but not at 0 h. There was no significant ($P>0.1$) bull by equilibration time interaction nor was there a significant observer effect for any response.

Table 3. Modified TALP Buffer

Ingredient	Concentration
NaCl	95.0 mM
KCl	3.0 mM
$NaHPO_4$	0.3 mM
$NaHCO_3$	10.0 mM
$MgCl_2 \bullet 6H_2O$	0.4 mM
Na Pyruvate	2.0 mM
Glucose	5.0 mM
Na lactate	25.0 mM
HEPES[a]	40.0 mM
Bovine serum albumin[b]	3.0 mg/ml
Gentamycin Sulfate	30.0μg/ml

[a] #H3375, Sigma Chemical Co., St. Louis, MO, USA

[b] #US70195, fraction V; Amersham/Life Science, Cleveland, OH, USA

Table 4. Effect of pre-freeze equilibration time on post-thaw progressive motility (%)

Equilibration at 5°C	Post-thaw incubation at 37°C		
	0 h	1 h	2 h
3 h	41[a]	36[a,b]	16
6 h	41[a]	37[a]	18
18 h	35[b]	31[b]	12
S.E.[c]	1.5	0.8	2.0

[a,b] Within columns, means without common superscripts differ ($P<0.05$), Tukey's HSD.

[c] Pooled standard errors,

$$\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$$

6. *Conclusion*. The results indicated no differences in post-thaw sperm motility between 3 and 6 h of total equilibration time at 5°C, but there was a significant decline in sperm motility following 18 h of equilibration at 5°C before freezing. The 3- to 6-h range permits pooling 2 consecutive 3-h sorting batches for freezing sperm without decreasing post-thaw motility.

As the bull by equilibration-time interaction was not significant, 3 to 6 h equilibration was adequate, with the caveat that only 4 bulls were used. The optimum equilibration time for a minority of bulls is expected to be >6 h.

Example 3

Effects Of Stain Concentration And Laser Power on Sorted Sperm

Objective: to evaluate the effects of Hoechst 33342 dye concentration in combination with laser intensity on flow-sorted sperm.

1. *Collection of Source Sample*. Sperm of 6 bulls were collected and prepared as described in Example 1A.

2. *Methods*.

a) *Experimental Design*. One ejaculate (2 bulls) and 2 ejaculates on different days (4 bulls) were used in a 2 by 2 design plus control.

b) *Staining and Sorting*. Staining, preparation for sorting and sorting sperm were achieved as described in Example 2 except that the Hoechst 33342 dye was added to sperm suspensions at a final concentration of 149 μM or 224 μM; and sperm were bulked-sorted with the laser operating at 100 mW or 150 mW of incident power. Bulk-sorted sperm were collected into 50-ml plastic tubes as described in Example 2. Four tubes containing approximately 15×10^6 total sperm/tube were collected over 1 h for each bull. The sorted sperm were incubated for 1 h at 22°C to simulate a longer sorting time.

c) *Preparation for Freezing*. Following incubation, the sperm were cooled as in Example 2. The sperm were then concentrated by centrifugation at 5°C at 850 × g for 20 minutes. After removing the supernatant, 150 μl of Tris-A fraction extender was added to each 150-μl sperm pellet at 5°C. All of the sperm pellets were suspended by gentle repeated aspiration and the sperm of individual bulls were pooled. Tris B-fraction extender was added stepwise as described in Example 2. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in Tris extender containing 6% glycerol and cooled to 5°C while the bulk-sorted sperm were being prepared.

d) *Equilibration and Freezing*. The control and sorted sperm were packaged into 0.25-ml polyvinylchloride straws as described in Example 2, equilibrated at 5°C for 3 h and then frozen conventionally.

3. *Evaluation of Post-Thaw Motility*. Straws were thawed and evaluated as described in Example 2.

4. *Statistical Analysis*. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via ANOVA. The model included dye concentration, laser intensity and bulls in the main plot, and observer and related interactions in the subplot. Bulls were considered a random effect and the other factors as fixed.

5. *Results*. Bull effects were significant for percentages of progressively motile sperm immediately after thawing ($P<0.1$) and after 1 h and 2 h of incubation at 37°C ($P<0.05$). There was no effect of dye concentration or bull by dye concentration on sperm motility at any incubation time. With bulls considered as a random effect, 150 mW of laser power resulted in lower post-thaw motility of sperm than 100 mW at 0 h of incubation ($P<0.1$), but not at other incubation times (Table 5). If bulls are considered as fixed effects, 150 mW of power resulted in lower sperm motility than 100 mW ($P<0.05$) at all 3 incubation times. There was an effect of bull by laser power ($P<0.05$) on sperm motility at 1 h, but not at 0-h or 2-h incubation times. Also, the higher laser power resulted in lower sperm motility than the control ($P<0.05$) at 0- and 1-h incubation times (Table 5). There was a significant observer effect at 1-h, but not at 0=h or 2-h, incubation times. There was no observer by treatment interaction ($P>0.1$).

Table 5. Effects of laser intensity and dye concentration on post-thaw motility (%).

	Incubation at 37°C		
Main effect means	0 h	1 h	2 h
Control	49	44	33
Dye Concentration			
149 µM	41	39	30
224 µM	42	39	30
Laser Intensity			
100 mW	46	42	33
150 mW	38[a]	35[b]	27
S.E.[c]	2.2	1.2	1.3

[a] Significant main effect ($P<0.1$) and differs from control ($P<0.05$).

[b] Differs from control ($P<0.05$).

[c] Pooled standard errors, $\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$

6. *Conclusion*. Percentages of progressively motile sperm post-thaw were diminished by the staining and sorting process. Higher laser intensity was more damaging than the lower laser intensity. There was no effect of dye concentration on post-thaw sperm motility. Thus, excitation of the sperm-bound Hoechst 33342 dye at lower laser intensities is less damaging and that staining sperm at the higher dye concentration had no detrimental effect on post-thaw motility. The damage observed was presumably to the sperm-motility apparatus.

Example 4

Evaluation of Pre-Sort Staining Procedures and

Selection of Extenders for the Cryopreservation Of Sperm

Objective: (1) to evaluate three pre-sort treatments for sperm; and, (2) and to evaluate sheath fluid and extender combinations for the cryopreservation of flow-sorted sperm.

The following experiment was replicated in its entirety:

1. *Collection of Source Sample*. Sperm from 4 bulls were collected and prepared as described in Example 1A.

2. *Methods*.

a) *Experimental Design*. A 3 (pre-sort treatments) by 3 (extenders) by 2 (sheath fluids) by 4 (bulls) by 2 (observers) factorial experiment was designed to determine the best procedure to hold sperm prior to sorting, and to evaluate three extenders for cryopreserving the sorted sperm. .

b) *Sample Preparation and Staining*. Freshly collected sperm from each of 4 bulls were treated as follows:

(1) diluted to 400×10^6 /ml in modified TALP (see Example 2, Table 3) and stained for 1 h at 34°C before bulk-sorting ("Dilute – 0 h");

(2) incubated neat at 22°C for 3 h before dilution, staining and sorting ("Neat – 3 h"); or,

(3) diluted and stained as "Dilute-0 h" and then incubated at 22°C for 3 h before bulk-sorting ("Diluted – 3 h").

c) *Extenders*. The following freezing extenders were compared: EYC (see Example 1) containing 7% glycerol, egg yolk-Tris (see Example 2) containing 6% glycerol, and egg yolk-TES-Tris (TEST) containing 5% glycerol. EYC "A Fraction" refers to the EYC extender containing no glycerol, and EYC "B Fraction" refers to EYC extender containing twice the final, desired glycerol

concentration (i.e., 14%). Thus, when EYC A and B fractions are combined in equal volume, the final EYC extender contains 7% glycerol. Tris A and B fractions are similarly named, and described in Example 2. TEST extender is prepared as a complete extender containing 5% glycerol; hence, there were no "A" and "B" fractions for TEST.

d) *Sheath Fluid*. Sheath fluid was either 98.6 mM sodium citrate dihydrate (#S279-3, Fisher Scientific, Fair Lawn, NJ) or Tris as described in Example 2. Both types of sheath fluid were adjusted to pH 6.8; osmolality was about 270 to 280 mOsm/kg. Tris sheath fluid was used to collect sperm that were later extended in egg yolk-Tris and TEST freezing extenders. Sheath fluid containing 98.6 mM sodium citrate dihydrate was used to collect sperm to later be extended in EYC freezing extender.

e) *Sorting*. Approximately 58×10^6 sperm for each combination of pre-sort treatment, sheath fluid and extender were bulk-sorted as described in Example 2 using 150 mW of incident laser power. For each sort, sperm were collected over approximately 1 h. After sorting, the samples were incubated at 22°C for 2 h to simulate a 3 h sort.

f) *Preparation for Freezing*. Following incubation, the sperm were cooled as described in Example 2. After cooling, the samples were centrifuged at 5°C at 850 × g for 20 min. Each sample comprised about 28 ml total volume and was contained in a 50-ml plastic tube

After the supernatant was removed, the sperm were returned to a 5°C cold room for extension. Samples were extended to 40×10^6/ml by depositing 131 µl of the sperm suspension into 69 µl of A-fraction EYC, A-fraction egg yolk-Tris, or TEST extender. Immediately, suspensions were adjusted to 20×10^6 sperm/ml with the addition of the matched glycerol containing extender (i.e., B-fraction EYC, B-fraction Tris) or TEST. B-fraction extenders were added to their respective samples stepwise (2X) at 15-min intervals as described in Example 2.

The TEST was added to sperm stepwise in the same manner as B-fraction EYC and Tris extenders.

g) *Equilibration and Freezing*. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for 3 h at 5°C and then frozen in static liquid nitrogen vapor.

3. *Evaluation of Post-Thaw Motility*. Thawing and post-thaw evaluations of sperm were done as described for Example 2.

4. *Statistical Analysis*. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each post-thaw incubation time. The main plot included pre-sort treatment, extenders, and bulls; the subplot consisted of observers and associated interactions. Bulls were considered a random effect, and the other factors, fixed. The entire experiment was replicated twice. Tukey's HSD test was used to separate means.

5. *Results*. Post-thaw progressive motility of bulk-sorted sperm was affected ($P<0.05$) by extender and bulls at each post-thaw incubation time and by pre-sort procedure at 0 h of incubation (Table 6). There were no differences due to sheath fluids ($P>0.05$). At 0-h post-thaw incubation, use of the neat-3h treatment resulted in more motile sperm after freezing and thawing than the other 2 pre-sort staining treatments ($P<0.05$; Table 6). However, pre-sort procedures were not statistically significant after post-thaw incubation of sperm for 1 or 2 h with bulls considered as a random effect. Importantly, at these 2 incubation times, there were significant pre-sort treatment by bull interactions ($P<0.05$). Furthermore, pre-sort treatment would have been a significant effect at all post-thaw incubation times had bulls been considered as fixed effects.

Immediately after thawing (0 h), TEST was the best extender, but after 1 or 2 h of incubation of 37°C, Tris was the best extender. Importantly, there was no pre-sort treatment by extender interaction for any response. There were observer effects ($P<0.01$) at all incubation

times, but no observer by treatment interactions. There was a bull by extender interaction ($P<0.05$) at all 3 incubation times.

Table 6. Main effects of pre-sort treatment and freezing extenders on post-thaw progressive motility (%)

		Incubation at 37°C		
Pre-sort procedure	Extender	0 h	1 h	2 h
Dilute – 0 h	Mean	39[a]	32	22
Neat – 3 h	Mean	43[b]	36	25
Dilute – 3 h	Mean	38[a]	31	19
Mean	EYC	36[a]	29[a]	17[a]
Mean	Tris	40[b]	39[b]	29[b]
Mean	TEST	44[c]	33[c]	20[a]
S.E.[d]		0.8	0.8	0.7

[a,b,c] Means within columns, within main effects, without common superscripts differ ($P<0.05$).

[d] Pooled standard errors $= \sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$

6. *Conclusion*. This study showed that holding sperm neat for 3 h before dilution, staining and sorting was better than immediate dilution and staining 0 h or 3 h later. Thus, by 3 h into the sort, it is best to continue with a new aliquot of the original ejaculate that was held neat 3 h and then stained, rather than continuing with the original sample of sperm stained and held at 400 x 10^6 sperm/ml.

Even though TEST extender provided higher post-thaw motility at 0 h, Tris was the superior extender when sperm were stressed by incubation at 37°C. Either sheath fluid worked equally well for each extender. Based on these results, we have incorporated the use of Tris sheath fluid in combination with Tris freezing extender into our standard operating procedure.

Example 5

Effects Of Extender Additives on Sorted Sperm

Objective: to evaluate the effect of adding sodium dodecyl sulfate ("SDS") to the freezing extender on flow-sorted sperm.

A. *Evaluation of Effect of Concentration of SDS in Freezing Extender*

1. *Collection of Source Sample*. Sperm of 6 bulls were collected and prepared as described in Example 1A.

2. *Methods*. Sperm from each of 6 bulls were extended to 20×10^6/ml in 20% whole egg Tris ("WET") extender containing 0, 0.03, 0.06, 0.09, or 0.12 percent SDS, packaged into straws and frozen. WET extender was prepared using 3.028 g of Tris[hydroxymethyl] aminomethane, 1.78 g of citric acid monohydrate, and 1.25 g of fructose per 100 ml of double distilled water, to which 20% whole egg (vol/vol) was added. The WET extender was prepared at a pH of about 7.0 and contained a final glycerol concentration of about 6% (vol/vol). The WET extender also contained 1000 IU of penicillin "G" sodium and 100 μg of streptomycin sulfate/ml.

3. *Results*. The respective means (n = 1 sample from each of 6 bulls) were 51, 51, 50, 51, and 48% progressive motile sperm approximately 10 minutes post-thaw. Based on these results, 0.06 percent SDS was used in Example 5B.

B. *Evaluation of the Effects of 0.06 Percent SDS in Various Freezing Extenders on Post-Thaw Motility of Flow-Sorted Sperm*

1. *Collection of Source Sample*. Sperm of 8 bulls were collected and prepared as described in Example 1A.

2. *Methods*. Post-thaw motility was studied for sperm frozen in egg yolk-Tris (see Example 2) and WET extenders (see Example 5A) with and without 0.06% SDS. Final glycerol content for both extenders was 6%.

a) *Staining, Preparation for Sort, Sorting*. Stained sperm samples were prepared from an ejaculate from each of 8 bulls as described in Example 2. Stained sperm were bulked-sorted using Tris sheath fluid as described in Example 2 except that the sort was achieved using 135 mW of incident laser power. Sorted sperm were collected in a 50-ml plastic tube containing 2 ml of A-fraction freezing buffer for each extender; 15×10^6 total sorted sperm (25 ml) for each treatment were collected and incubated for 1 h at 22°C to simulate longer sorting.

b) *Preparation for Freezing*. Diluted sperm were then cooled to 5°C over 90 minutes. An equal volume of appropriate B-fraction extender was added stepwise (2x) at 15-minute intervals to each 50-ml plastic tube containing sorted sperm. Aliquots of 25 ml/extender treatment were concentrated by centrifugation for 20 minutes at 850 x g in a refrigerated centrifuge. The supernatant was removed leaving a 600 µl sperm pellet, which was suspended by gentle vortexing for 15 seconds. No additional extender was added to the sperm pellet since the suspension containing the pellet already contained glycerol. The concentration of the sperm suspension was approximately 20×10^6/ml. A non-stained, non-sorted control for each bull was prepared at 20×10^6 sperm/ml in egg-yolk-Tris extender containing 6% glycerol. The control was placed in a 5°C cold room while bulk-sorting occurred.

c) *Equilibration and Freezing*. All control and bulk-sorted sperm were packaged and frozen at the same time. Sperm were packaged into 0.25-ml polyvinylchloride straws, equilibrated for about 3 h to about 6 h at 5°C and then frozen in static liquid nitrogen vapor.

3. *Evaluation of Post-Thaw Motility*. Thawing and post-thaw evaluations of sperm were done as described for Example 2 with the exception that progressive motility was evaluated 0.5 and 2.0 h after incubation.

4. *Statistical Analysis*. A general description of statistical analyses is provided in Example 2. Specifically, treatment effects were evaluated via separate analyses of variance for each incubation time; the model included bull and extender in the main plot and observer and

related interactions in the subplot. Differences in means were determined by the least significant difference test.

5. *Results*. Extender affected ($P<0.05$) progressive motility of sperm after 0.5 or 2 h post-thaw incubation (Table 7). At 0.5 h, WET plus SDS resulted in lower motility than Tris with SDS. At 2 h, all treatments with bulk-sorted sperm were worse than the non-sorted control sperm. There were significant bull and observer effects ($P<0.01$) at both incubation times, but no observer by treatment interactions.

Table 7. Effect of extender on post-thaw progressive motility (%)

	Incubation at 37°C	
Extender	0.5 h	2 h
Tris (non-sort)	42[a]	41[a]
Tris w/o SDS	40[a,b]	35[b]
Tris w/SDS	42[a]	37[b]
WET w/o SDS	40[a,b]	35[b]
WET w/SDS	38[b]	35[b]
S.E.[c]	1.0	1.2

[a,b] Means within columns without common superscripts differ ($P<0.05$).

[c] $\sqrt{error\ mean\ square\ of\ ANOVA} \div \sqrt{N}$

6. *Conclusion*. The inclusion of SDS in Tris or WET extenders did not benefit sperm quality as determined by visual estimates of motility after thawing. Also, results using WET and Tris extenders were similar; hence, WET appeared as efficacious as Tris for cryopreserving sorted bovine sperm.

Example 6

Quality of Sperm Sexed by Flow Sorting for Field Trials

Objective: to evaluate post-thaw quality of sorted sperm based on acrosomal integrity.

1. *Collection of Source Sample*. Sperm of 3 bulls were collected and prepared as described in Example 1A.

2. *Methods*. Sorted and non-sorted control sperm from the same ejaculate were stained, processed, and sorted as described in Example 2 except the sperm were sorted for sex-type at a 90% purity level. Sorted sperm were collected to a volume of approximately 20 ml and were cooled to 5°C for 90 minutes (0.2°C/min). After cooling, an equal volume of egg yolk-Tris B extender (see Example 2) was added to the sorted sperm in 2 equal volumes at 15-minute intervals. Centrifugation and aspiration of the supernatant were achieved as described in Example 5. After centrifugation and aspiration, egg yolk-Tris extender containing 6% glycerol (v/v) was added to the sperm pellet to bring the concentration of sperm to about 20 x 10^6 /ml. Freezing and thawing were done as described in Example 2 except that equilibration time was about 3 h.

3. *Evaluation of Post-Thaw Motility*. Visual estimates of the percentage of progressively motile sperm at 37°C were made approximately 10 minutes after thawing. The acrosomal integrity of sperm was assessed using differential interference-contrast microscopy (x1000) after 2 h of incubation at 37°C. Sperm were treated with 40 mM sodium fluoride, a wet was smear made, and 100 sperm per treatment were examined. Acrosomes were classified as: (a) intact acrosome, (b) swollen or damaged acrosome, or (c) missing acrosome (non-intact).

4. *Statistical Analysis*. The data analyzed were from 19 different freeze dates balanced across 3 bulls used in field trials. Treatment effects (sort vs. control) were evaluated via analysis of variance with bulls as a fixed effect.

5. *Results*. The percentage of progressively motile sperm post-thaw was significantly higher ($P<0.05$) for non-sorted sperm (50%) than for sorted sperm (46%; Table 8), despite removal of dead sperm during sorting. However, the percentage of sperm with an intact

acrosome was not different. Sorting increased the percentage of sperm missing an acrosome, but also reduced the percentage of sperm with a damaged acrosome, relative to control sperm ($P<0.05$). There were significant differences among bulls for percent of intact acrosomes ($P<0.05$), percent of non-intact acrosomes ($P<0.01$), and post-thaw progressive motility ($P<0.01$). There was a bull by sorting effect for post-thaw motility ($p<0.01$) but not for the other responses. From bulls A and B, differences in post-thaw motility between sorted and unsorted sperm were near zero; for bull C, sorted sperm were 10 percentage points (19%) lower in motility than control sperm.

Table 8. Effect of sorting on post-thaw motility (%) and acrosomal status (%).

	Acrosomal status			
	Intact	Damaged	Non-intact	Post-thaw motility
Control	64[a]	20[a]	15[a]	50[a]
Sorted	65[a]	14[b]	21[b]	46[b]

[a,b] Column means with different superscripts differ ($P<0.05$).

6. *Conclusion*. Visual estimates of progressive motility for sorted, frozen sperm on average were slightly lower (4 percentage points; 8%) than for control sperm, although this difference was larger for one bull. These evaluations were made approximately 10 minutes after thawing. The small average difference is consistent with that for non-intact acrosomes after 2 h of incubation. Sperm with a damaged or missing acrosome are likely to be immotile. The increased percentage of sperm with a non-intact acrosome, for sorted samples, indicates damage associated with sorting or with cryopreservation before or after actual sorting. Presumably, sorting converted damaged acrosomes to missing acrosomes. Based on standard procedures for evaluation of sperm quality, there is no basis for assuming that fertilizing potential of these flow-sorted sperm should be severely compromised for most bulls.

Example 7

Sex-Selection and Cryopreservation of Bull Sperm Using 20% Egg Yolk-Tris Extender

Objective: to provide a protocol for the cryopreservation of flow-sorted bull sperm.

1. *Collection and Ejaculate Assessment*. Collect and prepare ejaculates as described in Example 1A. Select ejaculates from those bulls with >75% morphologically normal sperm. Visually estimate the percentage of progressively motile sperm (ejaculates that have progressive motility >60% are best for sorting). Add antibiotics to raw semen as follows: tylosin at a final concentration 100 μg/ml, gentamicin at a final concentration of 500 μg/ml, and linco-spectin at a final concentration of 300/600 μg/ml.

2. *Staining and Preparation for Sort*. Following the addition of the antibiotics to the raw semen sample, allow 15-20 minutes before staining. Stain samples as described in Example 2.

3. *Sorting*. Sort for both X- and Y- type sperm, setting the sorting gates for 90% purity. Sort sperm into 50-ml Falcon tubes containing 2 ml 20% egg yolk-Tris A-fraction extender (see Example 2) until each tube contains a maximum of 20 ml total volume (or a maximum of 2 h per sort) and final sorted sperm concentration is 6×10^5/ml. Note that additional 20% egg yolk-Tris-A fraction catch buffer must be added after the sort and prior to cooling so that the final percentage of egg yolk is at least 3%.

4. *Preparation for Freezing*. Following the sort, cool the sorted samples to 5°C over a period of 90 minutes. After cooling, add 20% egg yolk-Tris B-fraction extender (see Example 2) stepwise (2X) at 15 minutes intervals. The final volume of Tris B-fraction extender added to the sperm sample should be equal to the volume of Tris A-fraction extender. The total volume of sperm sample after the Tris B-fraction extender is added should not exceed 27 ml total volume.

After the Tris B-fraction extender is added to the sperm sample, concentrate the sample by centrifugation for 20 minutes at 850 x g. Aspirate the supernatant leaving approximately 150 μl sperm pellet. Resuspend the sperm and pool the sperm for each individual bull.

5. *Freezing*. Add complete egg yolk-Tris extender (6% glycerol) to achieve a final sperm concentration of 20×10^6/ml. Package the extended sperm into 0.25-ml polyvinylchloride straws for freezing as described in Example 2.

Example 8

Evaluation of the Fertility of Flow-sorted, Frozen Bull Sperm in Field Studies

MATERIALS AND METHODS

Semen Collection and Processing

Semen from young bulls of unknown fertility was collected via artificial vagina (see Example 1A). After determining sperm concentration with a spectrophotometer and subjective evaluation of progressive sperm motility, semen was processed and sorted as described in Example 2 except that the sperm were sorted by sex-type at 90% purity using a laser incident power of about 135 to about 150 mW. Processing and freezing was achieved as in Example 2 except that the equilibration time was about 3 h. Cornell Universal Extender (Seidel GE Jr., Theriogenology 1997; 48:1255-1264) was used for liquid semen in field trials 1, 2, and 3. For frozen semen in field trials 2 and 3, the extender used was 2.9% Na citrate + 20% egg yolk with a final glycerol concentration of 7% (see Example 1). For field trials 4 through 11, sperm were frozen in a Tris-based extender composed of 200 mM Tris, 65 mM citric acid, 56 mM fructose, 20% egg yolk, and a final glycerol concentration of 6% (see Example 2). The sheath fluid used in the flow cytometer was 2.9% Na citrate (see Example 4) for trials 1, 2, and 3, and a Tris buffer for the remaining trials (see Example 2).

Sperm were packaged in 0.25-mi French straws in columns as small as 50 µl in the center of the straw. To minimize dilution effects, low volumes were used so there were at least 10^7 sperm/ml. In most trials, a column of extender without sperm was aspirated into the straw first to wet the cotton plug, followed by a small column of air, and then the sexed sperm. When sperm were frozen, one straw from each batch was thawed in 35°C water for 30 sec for quality control, and batches with less than 25% progressive motility post thaw were discarded. A sample of sexed sperm from each batch was sonicated and analyzed by flow cytometry to determine the accuracy of sexing.

Heifer Management and Artificial Insemination

The heifers used were in 6 widely scattered production units with different management practices. Seasonal and breed differences contributed further to the heterogeneity of the experiments (Table 9). Insofar as possible, treatments and controls were alternated systematically within bulls within inseminators as heifers entered the insemination facilities.

Estrus was synchronized in one of 4 ways (Table 9): (1) 500 mg of melengesterol acetate (MGA) fed daily in 2.3 kg of grain for 14 days followed by an i.m. injection of 25 mg prostaglandin $F_2\alpha$ (Lutalyse, Upjohn, Kalamazoo, MI, USA) 17, 18 or 19 days after the last day of feeding MGA (MGA/PG); (2) a single injection of 25 mg of prostaglandin $F_2\alpha$ (PG); (3) 20 or 25 mg of prostaglandin $F_2\alpha$ injected i.m. at 12-day intervals (PG/PG) or (4) 50 or 100 μg of GnRH injected i.m., followed by 25 mg of prostaglandin $F_2\alpha$ 7 days later (GnRH/PG).

Heifers were inspected visually for standing estrus mornings and evenings, but inseminated only in the evenings after 16:00, approximately ½ or 1 day after onset of estrus. Insemination was either into the uterine body conventionally, or half into each uterine horn using atraumatic embryo transfer sheaths (IMV, Minneapolis, MN, USA). In the latter case, semen was deposited past the greater curvature of the uterine horn as far anterior as could be accomplished without trauma, identically to nonsurgical embryo transfer. In most cases, semen was deposited between the anterior third and mid-cornua.

Most experiments included a frozen sperm control inseminated into the uterine body with 20 or 40 x 10^6 sperm/dose from the same bulls used for sperm sorted for sex-type ("sexed"). This control served as a composite estimate of the intrinsic, normal fertility of the heifers under the specific field-trial conditions as well as the fertility of the bulls used and the skills of the inseminators. Some trials also included a low-dose, unsexed control group. Sometimes numbers of control inseminations were planned to be ½ or ⅔ the number used for each treatment to obtain more information on sexed sperm. Frozen sexed and control sperm were thawed for 20 to 30 sec in a 35 to 37°C water bath. Various other details are summarized in Table 9.

Pregnancy was diagnosed by ultrasound 28 to 37 d post insemination and/or 56 to 92 d post-insemination, at which time fetal sex was determined in most trials, as described in Curran, S., Theriogenology 1991; 36:809-814, without the operator's knowing insemination treatments or controls. Sexes of calves born were nearly identical to the fetal-sex diagnosis. Data were

analyzed by single-degree-of-freedom Chi square corrected for continuity; 2-tail tests were used unless 1-tail is specified. Fewer than 5% of the inseminations were culled due to errors of insemination treatment, frank infection of the reproductive tract, failure to traverse the cervix, etc. Decisions to cull animals from experiments were made shortly after insemination and were never based on the pregnancy diagnosis.

Table 9. Procedural details of field trials

Trial	Insemination dates	Breeds of heifers	Bulls used	Inseminators	Estrus synchronization	Comments
1	5/20-23, 1997	Angus	N1, N2, AN4	A, B	MGA/PG	Included low-dose controls
2	2/18-5/22, 1998	Angus crossbred	N3, N4, N5, N6	C, D	PG/PG	Low dose but no normal-dose controls; some heifers pregnant and aborted when synchronized
3	6/2-6/5, 1998	Angus	AN4, AN5, N7, N8	B, D	MGA/PG	
4	2/10-13, 1999	Holstein	J2, J4	C, D	PG	Very severe mud, snow, wind, and cold, driving rain
5	2/24-26, 1999	Holstein	J2, J4, J5	C, B, D	PG/PG	
6	4/14-16, 1999	Holstein	J2, J3, J4, J5	C, D	PG	Some heifers were reproductive culls
7	4/27-5/1, 1999	Hereford& Angus crossbred	AN1, AN4	C	MGA/PG	Semen for 1 bull shipped 6 h before sorting; severe weather
8	4/21-23, 1999	Angus crossbred	H1, H2	E	MGA/PG	Feedlot heifers
9	5/5-8, 1999	Red Angus	AR1, AR2	C, F	MGA/PG	
10	5/31-6/2, 1999	Angus	AN4, AN7, AN8	B, D	GnRH/ PG	
11	7/28-30, 1999	Holstein	H2, H3	C, D	PG/PG	First replicate available in a much larger trial

RESULTS AND DISCUSSION

The data presented are from 11 consecutive, heterogeneous field trials, constrained by logistical aspects of the studies, such as having to match bulls to genetic needs of the herds, unavailability of fertility information on bulls, limited numbers of heifers, unavailability of the same inseminators across trials, severe weather in some trials, limited amounts of sexed semen in early trials, 2 sets of heifers in which some turned out to be pregnant up to about 55 days at the time of estrus synchronization, etc. Up to 4 bulls and 3 inseminators were involved with each trial; this enabled us to sample populations to ensure that results applied to more than one bull or technician; however, insufficient data were produced to evaluate bull-to-bull differences in fertility rigorously.

Most sets of heifers were from breeding herds located 140 to 250 km from our laboratory. There were no significant differences in pregnancy rates between inseminators in any trial, but numbers of breedings per inseminator were low, and differences likely would be detected with larger numbers of inseminations.

Estrus synchronization methods were not compared within trials, so it was not possible to compare pregnancy rates among these methods. Pregnancy rates appeared to be satisfactory for all four synchronization procedures used.

Since inseminations were done once a day, heifers in estrus evenings were inseminated approximately 24 h after estrus was detected. The pregnancy rate for these heifers with sexed sperm pooled over all trials was 203/414 (49.0%), which was not significantly different ($P>0.1$) from that of heifers in estrus mornings and thus inseminated half a day after estrus detection 266/586 (45.4%). This tendency for higher fertility with later insemination is in agreement with findings from other research that it is preferable to inseminate later than normally recommended with lower fertility bulls, when low sperm numbers are used, or when conditions are otherwise suboptimal.

Pregnancy rates by treatments and, when available, fetal or calf sex are presented in Tables 10 to 20. The objective was to obtain female offspring, except in trial 8; accuracy was 95%, 83%, 90%, 83%, 82%, and 94% in Trials 1, 3, 8, 9, 10, and 11, respectively. In the remainder of the trials, fetal or birth sexes were not available because of timing of pregnancy diagnosis, unavailability of persons skilled in sexing fetuses, and/or because calves have not yet

been born. This was not a major concern because the main objective of this research was to determine fertility of flow-sorted sperm inseminated at low doses.

The accuracy of sexing can be adjusted to virtually any level desired between 50 and 95+% by adjusting the sorting parameters. However, higher accuracy results in lower numbers of sperm sorted per unit time, particularly for Y-chromosome sperm. 90% accuracy is sufficient for routine work.

The main findings from each field trial will be summarized in turn. Note that total sperm numbers are given in table headings; numbers of progressively motile sperm usually were 30 to 50% of these values. Field trial 1 (Table 10) confirmed that pregnancy rates with uterine horn insemination using low numbers of unsexed sperm were similar to controls with normal sperm numbers. The day 64 to 67 pregnancy rate with unfrozen sexed sperm (42%) was 12 percentage points below the unsexed liquid control with sperm diluted, stained, and centrifuged identically to the sorted sperm. Accuracy of sexing was 95%; the sex of calves born from sexed sperm matched the sex diagnosis of fetuses exactly; there was one mistake in sexing fetuses of controls. There were no abortions between 2 months of gestation and term, and all 19 calves from the sexed sperm treatment were normal and survived. For the sexed semen treatment, the 2-month pregnancy rates for bulls N1, N2, and N3 were 41, 44, and 40%, respectively; 39% (13/33) of heifers in estrus in the morning and 50% (6/12) in estrus in the evening became pregnant.

Table 10. Results of field trial 1 -- Angus heifers in Wyoming, 1997

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 33	No. pregnant day 64 to 67	No. ♀ calves
Sexed, 5°C/horns	3×10^5	45	20 (44%)	19 (42%)	18 (95%)[a]
Control, 5°C/horns	3×10^5	28	15 (54%)	15 (54%)	5 (53%)[b]
Frozen, control/body	40×10^6	29	16 (55%)	15 (52%)	11 (73%)[a,b]

[a,b] Sex ratios without common superscripts differ ($P<0.02$).

Field trial 2 (Table 11) provided the first evidence that results with sexed, frozen sperm are similar to sexed, unfrozen sperm if adjustment is made for numbers of sperm killed during cryopreservation. There also was no difference in pregnancy rates between sexed sperm stored at 5 versus 18°C. Pregnancy rates at 2+ months after insemination for sexed semen from individual bulls ranged from 22 to 42% pregnant (P>0.05). Embryonic loss between 1 and 2 months of gestation was very similar for sexed and control pregnancies. Calving data were available from 39 heifers from this trial; each of these heifers (30 sexed pregnancies, 9 controls) pregnant at 2 months calved after a normal-length gestation.

Table 11. Results of field trial 2--Crossbred beef heifers in Colorado, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 35[a]	No. pregnant day 59 to 92[a]
Control, 5°C/horns	5×10^5	58	27 (47%)	24 (41%)
Sexed, 5°C/horns	5×10^5	51	17 (33%)	16 (31%)
Sexed, 18°C/horns	5×10^5	46	16 (35%)	12 (26%)
Sexed, frozen/horns	1×10^6	87	29 (33%)	28 (32%)

[a] No significant differences, χ^2.

Field trial 3 (Table 12) confirmed that sexed, frozen sperm results in reasonable pregnancy rates. The accuracy of sexing sperm was confirmed again; however, there were 4 errors in sexing fetuses relative to the calves born; the actual sexes of calves born are presented. Again, there were no abortions between 2 months of gestation and term. Pregnancy rates averaged over sexed, unfrozen and sexed, frozen sperm for bulls N8, N9, AN5, and AN4 were 24, 31, 50, and 60%, respectively (P<0.1).

Table 12. Results of field trial 3 -- Angus heifers in Wyoming, 1998

Treatment/site	No. sperm	No. heifers	No. pregnant day 62 to 65	No. ♀ calves
Sexed, 18°C/horns	5×10^5	37	11 (30%)[a]	10 (91%)[c]
Sexed, frozen/horns	1×10^6	35	18 (51%)[a,b]	14 (78%)[c]
Frozen, control/body	40×10^6	37	27 (73%)[b]	16 (59%)[d]

[a,b] Means without common superscripts differ P<0.05).
[c,d] The percentage of ♀ calves from the sexed treatments (83%) differed from the control group, P<0.05, 1-tail, χ^2.

Field trials 4, 5, and 6 (Tables 13, 14, 15) were done at the same location with 3 different groups of heifers. Unfortunately, it was not possible to replicate each trial similarly due to vagaries of field trials, such as scheduling personnel, availability of sexed semen from each bull, etc. The widely different pregnancy rates between trials 5 and 6 illustrate that conditions were different among trials. Some of the heifers in trial 6 were available because they failed to get pregnant after a month of natural mating. Under conditions of these trials, pregnancy rates were very similar between 1.5 and 3.0×10^6 sexed, frozen sperm/dose. Furthermore, there was no advantage to uterine-horn insemination. There were no significant differences (P>0.05) in pregnancy rates among bulls except in Trial 5 in which the pregnancy rate of J2, 20/28 (71%), was higher than that of J4, 15/39 (38%) (P<0.05). This difference was not consistent from trial to trial, as J4 had numerically but not significantly (P>0.1) higher pregnancy rates than J2 in Trials 4 and 6.

Table 13. Results of field trial 4 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 30 to 33	No. pregnant day 64 to 67*
Sexed, frozen/body	1.5×10^6	55	36 (65%)[a,b]	36 (65%)[a,b]
Sexed, frozen/body	3×10^6	52	27 (52%)[a]	26 (50%)[a]
Control, frozen/body	20×10^6	55	45 (82%)[b]	43 (78%)[b]

[a,b] Means without common superscripts differ (P<0.01).
* Six heifers pregnant at d 30 to 33 were sold before the second pregnancy diagnosis; these were assumed to have remained pregnant.

Table 14. Results of field trial 5 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 35[a]	No. pregnant day 60 to 62[a]
Sexed, frozen/body	1.5×10^6	23	12 (52%)	12 (52%)
Sexed, frozen/body	3.0×10^6	25	15 (60%)	14 (56%)
Sexed, frozen/horns	1.5×10^6	25	15 (60%)	12 (48%)
Sexed, frozen/horns	3.0×10^6	25	17 (68%)	15 (60%)
Control, frozen/body	20×10^6	30	20 (67%)	19 (63%)

[a] No significant differences.

Table 15. Results of field trial 6 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 31 to 34	No. pregnant day 60 to 63
Sexed, frozen/body	1.5×10^6	27	11 (41%)[a]	9 (33%)[a]
Sexed, frozen/body	3.0×10^6	25	10 (40%)[a]	9 (36%)[a]
Sexed, frozen/horns	1.5×10^6	24	8 (33%)[a]	7 (29%)[a]
Sexed, frozen/horns	3.0×10^6	24	10 (42%)[a]	8 (33%)[a]
Control, frozen/body	20×10^6	24	18 (75%)[b]	17 (71%)[b]

[a,b] Means without common superscripts differ ($P<0.05$).

For trial 7 (Table 16), only one inseminator was available due to rescheduling. This is the only trial that showed a convincing advantage of uterine-horn over uterine-body insemination. For this inseminator under the conditions of the trial, 55% more heifers (22 percentage points) became pregnant with sexed, frozen semen inseminated into the uterine horns than into the uterine body. The true difference could be smaller because there are wide confidence intervals on these means. In all the other trials (5, 6, 9, and 11) in which body- and horn-insemination were compared, pregnancy rates were very similar for both methods for this technician as well as for other technicians.

Semen from one of the bulls used in Trial 7 was shipped without dilution from Montana by air in an insulated box at ~20°C before sorting; shipping time was 6 h. Pregnancy rates for the sexed sperm from the two bulls were virtually identical, 49% for the unshipped and 52% for the shipped semen. Semen was not diluted with extender and not cooled for shipping because staining properties of sperm with Hoechst 33342 are altered by dilution with extenders. Furthermore, in other studies (see Example 4), storing semen neat at ambient temperature between collection and flow-sorting was found to be superior to diluting it.

Table 16. Results of field trial 7 -- Crossbred beef heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 33 to 37
Sexed, frozen/body	1.5×10^6	86	34 (40%)[a]
Sexed, frozen/horns	1.5×10^6	86	53 (62%)[b]
Control, frozen/body	20×10^6	35	18 (51%)[a,b]

[a,b] Means without common superscripts differ ($P<0.01$).

Field trial 8 (Table 17) concerned feedlot heifers not implanted with growth promotants; at the time pregnancy was diagnosed they were aborted, so calving data was not available. This experiment illustrates that efficacious sexing also can be done in the male direction. Pregnancy rates for the 2 bulls were 50 and 61%.

Table 17. Results of field trial 8 -- Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant[a] day 74 to 76	No. ♂ fetuses
Sexed, frozen, 72 mW laser/body	1×10^6	18	7 (39%)	6 (86%)
Sexed, frozen, 135 mW laser/body	1×10^6	18	13 (78%)	12 (92%)

[a] No significant differences.

Field trial 9 (Table 18) was the only trial to show a convincing advantage of 3.0 versus 1.5×10^6 sexed, frozen sperm/insemination dose. This advantage was true for both inseminators. Pregnancy rates for sexed sperm from the 2 bulls were 62 and 75%.

Table 18. Results of field trial 9 -- Red Angus heifers in Nebraska, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 60 to 63[a]	No. ♀ fetuses
Sexed, frozen/body	1.5×10^6	15	8 (53%)	7 (88%)
Sexed, frozen/body	3.0×10^6	14	12 (86%)	9 (75%)
Sexed, frozen/horns	1.5×10^6	16	9 (56%)	7 (78%)
Sexed, frozen/horns	3.0×10^6	16	12 (75%)	11 (92%)
Control, frozen/body	20×10^6	30	21 (70%)	13 (62%)

[a] 3.0×10^6 sexed sperm had a higher pregnancy rate (80%) than 1.5×10^6 sexed sperm (55%), $P<0.05$, 1-tail χ^2.

Pregnancy rates in field trial 10 (Table 19) with sexed, frozen semen, were similar to controls; the accuracy of sexing sperm on this trial was only 82%, which, however, is not significantly different from the targeted 90% accuracy. Pregnancy rates for sexed semen were 54, 66, and 50% for bulls AN4, AN7, and AN8, respectively ($P>0.1$). Eighteen of the heifers inseminated in this trial were the calves resulting from sexed sperm in field trial 1.

Table 19. Results of field trial 10 -- Angus heifers in Wyoming, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 61 to 63[a]	No. ♀ fetuses
Sexed, frozen/body	1×10^6	44	26 (59%)	23 (85%)
Sexed, frozen/body	3×10^6	43	23 (53%)	17 (74%)
Control, frozen/body	20×10^6	35	20 (57%)	12 (57%)

[a] No significant differences.

Table 20. Results of field trial 11 -- Holstein heifers in Colorado, 1999

Treatment/site	No. sperm	No. heifers	No. pregnant day 28 to 30[a]	No. pregnant day 56 to 58[a,b]
Sexed, frozen/body	1×10^6	12	8 (67%)	7 (58%)
Sexed, frozen/body	3×10^6	12	6 (50%)	4 (33%)
Sexed, frozen/horns	1×10^6	7	4 (57%)	4 (57%)
Sexed, frozen/horns	3×10^6	7	4 (57%)	4 (57%)
Control, frozen/body	20×10^6	9	4 (44%)	3 (33%)

[a] No significant differences, χ^2.
[b] 16 of 17 (94%) fetuses from the sexed semen treatments were female; 2 were too deep in the body cavity to sex with ultrasound.

Data from trials were combined in which treatments were identical except 1 x 10 and 1.5 x 10^6 sperm doses were pooled (Table 21).

Table 21. Meta-summary from combining trials with sexed, frozen semen and frozen controls.

Trials combined	Sperm no./site	No. heifers	No. pregnant
5, 6, 9, 11	1.0 - 1.5 x 10^6/body	77	36 (47%)
	3.0 x 10^6/body	76	38 (50%)
	1.0 - 1.5 x 10^6/horns	72	32 (44%)
	3.0 x 10^6/horns	72	39 (54%)
	20 x 10^6/body, control	93	61 (66%)
4, 5, 6, 9, 10, 11	1.0 - 1.5 x 10^6/body	176	98 (56%)
	3.0 x 10^6/body	171	88 (51%)
	20 x 10^6/body, control	183	124 (68%)
5, 6, 7, 9, 11	1.5 x 10^6/body	163	70 (43%)
	1.5 x 10^6/horn	158	85 (54%)
	20 x 10^6/body, control	128	79 (62%)

Pregnancy rates with sexed sperm were generally 70-90% of unsexed controls within experiments with 7 to 20 times more sperm. This difference was less in the more recent trials, possibly reflecting improved sexing and sperm-processing procedures.

In some trials, heifers were examined for pregnancy by ultrasound at both 1 and 2 months after insemination. Pregnancy losses in this interval were similar (P>0.1) for sexed (23/261; 8.8%) versus control (9/145; 6.2%) sperm treatments, which is one measure that genetic damage due to sexing is minimal. Calving information was obtained from only a few of the pregnant heifers because most cattle from the earlier trials were sold, and those from later trials have not calved yet. The population of calves produced to date from sexed semen appears to be no different from the population of controls.

CONCLUSION

Sex ratios in cattle can be distorted to about 90% of either sex by sorting sperm on the basis of DNA content with a flow cytometer/cell sorter followed by cryopreservation and relatively routine artificial insemination. Calves resulting from sexed sperm appear to be normal. For most bulls in these studies, pregnancy rates with 1.0 to 1.5 x 10^6 sexed, frozen sperm were 70 to 90% of unsexed controls with 20 or 40 x 10^6 frozen sperm inseminated conventionally. These results apply to well-managed heifers bred by well-trained inseminators using properly processed semen. There may be a small advantage to inseminating sexed sperm bilaterally into the uterine horns compared to standard uterine body insemination.

****

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

All patents and publications described are herein incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method for the cryopreservation of sperm comprising:

(a) obtaining a selected sperm sample;

(b) cooling said selected sperm sample;

(c) isolating sperm from said selected sperm sample to produce isolated sperm;

(d) adding final extender to said isolated sperm to produce a suspension of sperm; and

(e) freezing said suspension of sperm.

2. The method of Claim 1 wherein said selected sperm sample comprises a portion of the sperm present in a source sample, said portion of sperm selected for a characteristic, and wherein the sperm concentration in the selected sperm sample is lower than in the source sample.

3. The method of Claim 1 wherein said selected sperm sample comprises sex-selected sperm.

4. The method of Claim 1 wherein said selected sperm sample comprises mammalian sperm.

5. The method of Claim 4 wherein said selected sperm sample comprises bovine sperm.

6. The method of Claim 4 wherein said selected sperm sample comprises equine sperm.

7. The method of Claim 4 wherein said selected sperm sample comprises porcine sperm.

8. The method of Claim 1 wherein said selected sperm sample comprises sperm selected by a method from the group consisting of flow cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a biochemical technique, a technique based on

motility of sperm, a technique based on an electrical property of sperm, and any combination thereof.

9. The method of Claim 8 wherein said sperm have been selected by flow cytometry.

10. The method of Claim 1 wherein cooling is carried out by reducing the temperature of the selected sperm sample to about 5°Celsius.

11. The method of Claim 10 wherein cooling is carried out over a period of about 60 minutes to about 240 minutes.

12. The method of Claim 1 wherein the final extender added to said selected sperm sample each comprise, in addition to a cryoprotectant, one or more of the following components: a component that maintains osmolality and buffers pH, an organic substance that reduces cold shock and preserves fertility of sperm, an energy source, a substance that facilitates sperm capacitation, and an antibiotic.

13. The method of Claim 12 wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any combination thereof.

14. The method of Claim 12 wherein said cryoprotectant is selected from the group consisting of glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

15. The method of Claim 12 wherein said component that maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

16. The method of Claim 12 wherein said component that maintains osmolality and buffers pH is selected from the group consisting of sodium citrate, Tris[hydroxymethyl]aminomethane,

N-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

17. The method of Claim 12 wherein said organic substance is selected from the group consisting of egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.

18. The method of Claim 12 wherein said energy source is a monosaccharide selected from the group consisting of glucose, fructose, mannose, and any combination thereof.

19. The method of Claim 12 wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

20. The method of Claim 1 wherein, after the addition of the final extender, the sperm sample and suspension of sperm, respectively, comprise glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

21. The method of Claim 1 wherein, after the addition of the final extender, said sperm sample and suspension of sperm, each comprise glycerol, sodium citrate, egg yolk, and one or more antibiotics.

22. The method of Claim 1 wherein, after the addition of the final extender, said sperm sample and suspension of sperm, each comprise glycerol, egg yolk, milk, fructose, and one or more antibiotics.

23. The method of Claim 1 wherein said extender has a pH in the range of about 6.5 to about 7.5.

24. The method of Claim 1 wherein the sperm are isolated from said selected sperm sample by centrifugation.

25. The method of Claim 24 wherein said centrifugation allows for at least about 50% to about 90% recovery of sperm.

26. The method of Claim 1 wherein the concentration of sperm in said suspension prior to freezing is about 1 x 10^6/ml to about 300 x 10^6/ml.

27. A frozen selected sperm sample comprising a portion of the sperm present in a source sample, said portion of sperm selected for a characteristic.

28. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample comprises sex-selected sperm.

29. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample comprises mammalian sperm.

30. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises bovine sperm.

31. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises equine sperm.

32. The frozen selected sperm sample of Claim 29 wherein said frozen selected sperm sample comprises porcine sperm.

33. The frozen selected sperm sample of Claim 27 wherein the method used to select said selected sperm sample comprises a technique from the group consisting of flow cytometry, a magnetic technique, a columnar technique, a gravimetric technique, a biochemical technique, a technique based on motility of sperm, a technique based on an electrical property of sperm, and any combination thereof.

34. The frozen selected sperm sample of Claim 33 wherein said frozen selected sperm sample comprises sperm that have been selected by flow cytometry.

35. The frozen selected sperm sample of Claim 27 wherein said frozen selected sperm sample is produced by a method comprising:

(a) obtaining a selected sperm sample;

(b) cooling said selected sperm sample;

(c) isolating sperm from said selected sperm sample to produce isolated sperm;

(d) adding final extender to said isolated sperm to produce a suspension of sperm; and

(e) freezing said suspension of sperm.

36. A method comprising using the frozen selected sperm sample of Claim 27 for artificial insemination or in vitro fertilization.

37. The method of Claim 36 comprising using said frozen selected sperm sample for low-dose artificial insemination.

add A1

add B2

ABSTRACT OF THE INVENTION

The present invention provides a method of cryopreserving sperm that have been selected for a specific characteristic. In a preferred embodiment, the method is employed to freeze sex-selected sperm. Although the cryopreservation method of the invention can be used to freeze sperm selected by any number of selection methods, selection using flow cytometry is preferred. The present invention also provides a frozen sperm sample that has been selected for a particular characteristic, such as sex-type. In preferred embodiments, the frozen sperm sample includes mammalian sperm, such as, for example, human, bovine, equine, porcine, ovine, elk, or bison sperm. The frozen selected sperm sample can be used in a variety of applications. In particular, the sample can be thawed and used for fertilization. Accordingly, the invention also includes a method of using the frozen selected sperm sample for artificial insemination or in vitro fertilization.

Listing of Claims:

Claims 1-37 (Canceled)

38. (Previously Presented)

A method of cryopreserving sex-selected sperm cells, comprising:

a. obtaining sperm cells from a species of a male mammal;

b. sorting said sperm cells, without the presence of protective compounds in seminal plasma, and based upon sex-type to create a collection of sex-selected sperm cells;

c. cooling said sex-selected sperm cells;

d. suspending said sex-selected sperm cells in an extender to at least about 5 million per milliliter of extender to at least about 10 million per milliliter of extender, and;

e. freezing said sex-selected sperm cells in said extender.

39. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal are selected from the group consisting of bovine sperm cells and equine sperm cells.

40. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells between about 300,000 and about 3,000,000.

41. (Withdrawn)

A method of cryopreserving sperm cells as described in claim 39, and further comprising the step of isolating a number of bovine sperm cells of no more than about 1,000,000.

42. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said sperm cells from said species of said male mammal comprise equine sperm cells.

43. (Currently amended)

A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells between about 1,000,000 ~~million~~ and about 25,000,000.

44. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 42, and further comprising the step of isolating a number of equine sperm cells of no more than about 5,000,000.

45. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said step of cooling sex-selected sperm cells comprises reducing the temperature of said sex- selected sperm cells to about 5°Celsius.

46. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 45, wherein said step of reducing the temperature of said sex-selected sperm cells comprises reducing the temperature of said sex-selected sperm cells for a period of about 60 minutes to about 240 minutes.

47. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein said extender further comprises a component which maintains osmolality and buffers pH.

48. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein

said component which maintains osmolality and buffers pH is selected from the group consisting of a buffer comprising a salt, a buffer containing a carbohydrate, and any combination thereof.

49. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, wherein said component which maintains osmolality and buffers pH is selected from the group consisting of sodium citrate,Tris[hydroxymethyl]aminomethane, 200mM Tris[hydroxymethyl]aminomethane, 175 mM to 225mM Tris[hydroxymethyl] aminomethane, 200 mM Tris[hydroxymethyl]aminomethane/65mM citric acid monohydrate, 175 mM to 225mM Tris[hydroxymethyl]aminomethane/50mM to 70mM citric acid monohydrate, N-Tris [hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, 200 mM Tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid/65 mM citric acid monohydrate, 175 mM to 225 mM Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid/50mM to 70 mM citric acid monohydrate, monosodium glutamate, milk, HEPES buffered medium, and any combination thereof.

50. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47, 48, or 49, wherein said extender has a pH in the range of about 6.5 to about 7.5.

51. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 50, wherein said extender further comprises a cold shock protectant.

52. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said cold shock protectant is selected from the group consisting of egg yolk, 20% egg

yolk, 15% to 25% egg yolk, an egg yolk extract, milk, a milk extract, casein, albumin, lecithin, and any combination thereof.

53. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 51, wherein said extender further comprises an energy source.

54. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said energy source is selected from the group consisting of a saccharide, glucose, fructose, 56 mM fructose, 45mM to 60mM fructose, mannose, and any combination thereof.

55. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 53, wherein said extender further comprises an antibiotic.

56. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 55, wherein said antibiotic is selected from the group consisting of tylosin, gentamicin, lincomycin, linco-spectin, spectinomycin, penicillin, streptomycin, and any combination thereof.

57. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 47,51, 53, or 55, wherein said extender further comprises a cryoprotectant.

58. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of disaccharides, trisaccharides, and any conbination thereof.

59. (Previously Presented)

A method of cryopreserving sex-selected sperm cells as described in claim 57, wherein said cryoprotectant is selected from the group consisting of glycerol, 6% glycerol, between 5% to 7% glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, and any combination thereof.

60. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, Tris[hydroxymethyl]aminomethane, egg yolk, fructose, and one or more antibiotics.

61. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, sodium citrate, egg yolk, and one or more antibiotics.

62. (Withdrawn)

A method of cryopreserving sex-selected sperm cells as described in claim 38, wherein the extender in which said sex-selected sperm cells are suspended comprises glycerol, egg yolk, milk, fructose, and one or more antibiotics.

63. (Currently amended)

A method of cryopreserving sex-selected sperm cells as described in claim 38, further comprising ~~the step~~ a step of equilibrating said sex-selected sperm cells suspended in said extender to a cooler, non-freezing temperature for a period of time prior to said freezing step for a period of about 1 hour to about 18 hours.

64. (Currently amended)

A method of cryopreserving sex-selected sperm cells as described in claim 59, further comprising ~~the step~~ a step of equilibrating said sex-selected sperm cells suspended in said

extender to a cooler, non-freezing temperature for a period of time prior to said freezing step over a period of not greater than 6 hours.

65. (Withdrawn)
A frozen sex-selected sperm sample in accordance with the method of claim 38.

66. (Canceled)

67. (Withdrawn)
A method of cryopreserving sperm cells as described in claim 38, wherein said step of freezing said sex-selected sperm cells in said extender comprises freezing a number of bovine sperm cells between about 300,000 and about 5,000,000.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/478,299	01/05/2000	John L. Schenk	22091-701	1509

7590 05/13/2005

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EXAMINER	
MELLER, MICHAEL V	
ART UNIT	**PAPER NUMBER**
1654	

DATE MAILED: 05/13/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	09/478,299	SCHENK, JOHN L.	
	Examiner	Art Unit	
	Michael V. Meller	1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *28 February 2005*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *38-65 and 67* is/are pending in the application.

4a) Of the above claim(s) *40,41,55,56,60-62,65 and 67* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *38, 39, 42-54, 57-59, 63, 64* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1)☐ Notice of References Cited (PTO-892)
2)☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3)☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4)☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5)☐ Notice of Informal Patent Application (PTO-152)
6)☐ Other: _____.

DETAILED ACTION

Election/Restrictions

The election/restriction of record is maintained.

Claims 40, 41, 55, 56, 60-62, 65, 67 are withdrawn from further consideration as being drawn to non-elected subject matter.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim Rejections - 35 USC § 112

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

There is still no support in the specification for the ranges of sperm cells claimed. Applicant has not addressed this rejection but it is still valid. There is no support for limitations such as, "to at least about 5 million per milliliter to at least about 10 million

per milliliter of extender", "equine sperm cells between about 1,000,000 million and about 25,000,000".

The specification may mention specific points of the concentration (i.e. 1 $X10^6$) but it does not provide for the claimed range of the concentrations (i.e. between about 1,000,000 million and about 25,000,000). The concentrations in table 1 are noted but they are only isolated concentrations, there is no support for ranges of these concentrations, only the specific points tested.

The time limitation still has no support. In fact, as applicant noted, they have support for 1-18 hours, which clearly is more than 6 hours.

Applicant points to the specification at page 17, line 6, but this is not what the problem is. There is no support for "over a period of not more than 6 hours". This is because 1-18 hours clearly is more than 6 hours.

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 63 and 64 are still confusing. The way the applicant has now amended the claims is confusing since it is not clear how the "at least some of said sex selected sperm cells" can be not frozen as in claims 63 and 64 and indeed frozen in the main claim, claim 38. If claim 38 requires that the "at least some of said sex selected sperms cells" are to be frozen then claims 63 and claim 64 fail to further limit claim 38.

It might be clearer if applicant states in claims 63 and 64 that the steps of claims 63 and 64 are performed between steps d) and e) of claim 38.

Further, what is meant by , "to at least about 5 million per milliliter to at least about 10 million per milliliter of extender" ? Is this the concentration of the extender before it is applied to the sperm cells ? Is the 5 million referring to the number of sperm cells. So how much extender is added to each sperm cell ?

This was never addressed by applicant.

Cooling is still not clear in claim 38. Cooling to what temperature ?

Claims 63 and 64 are confusing. First there is no antecedant basis for "the step of equilibrating at least some of said sex-selected sperm cells". What step is this ? Second, what temperature is cooler ? Cooler than what ? This is relative and subjective. There is no point of reference for one to compare it to.

Claim Rejections - 35 USC § 103

Claims 38, 39, 42-54, 57-59, 63, 64 are rejected under 35 U.S.C. 103(a) as being unpatentable over Salisbury et al. in view of Spaulding and Shrimpton.

The claims still are obvious over the cited prior art of record. Applicant's limitation in claim 38 of "sorting said sperm cells, without the presence of protective compounds in seminal plasma" is met since as applicant notes on page 10 of their latest response filed 2/28/2005, that flow cytometry which is used by Spaulding has been shown to work and is now in practice as applicant has also done. Applicant states on page 10 that sorting

without the presence of protective compounds is a feature that occurs when flow cytometry is used to achieve sex sorting which Spaulding has also done just like applicant and this is of record. Thus, the references meet the claim limitations.

Applicant also argues that the concentrations of the sperm cells in the extenders is 5 million to 10 million and applicant has pointed to table 1 of the specification to support this, but these points go up and down. For example, the values go up as one approaches 10 million and then decreases as one goes above 10 million. Also, it is not clear what this signifies. It is not clear what significance the increase in the numbers means. Does this increase anything ? Just because one yields higher numbers at 10 million this does not correlate to anything and if it does what does it correlate to ? Further, it is not clear what the 61 refers to in relation to 10 million in table 1. Also it is noted that the values at 24 hours and 48 hours differ in some way. It is not clear what this signifies.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any

extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Michael V. Meller whose telephone number is 571-272-0967. The examiner can normally be reached on Monday thru Thursday: 9:30am-6:00pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Bruce Campell can be reached on 571-272-0974. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Michael V. Meller

Primary Examiner
Art Unit 1654

MVM

SECOND EDITION

Physiology of Reproduction and Artificial Insemination of Cattle

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SAN FRANCISCO

Library of Congress Cataloging in Publication Data

Salisbury, Glenn Wade, 1910-
Physiology of reproduction and artificial insemination of cattle.

(A Series of books in agricultural science: Animal Science) Includes index.
1. Cattle—Reproduction. 2. Cattle—Artificial insemination. I. VanDemark, N.L., joint author. II. Lodge, James Robert, 1925- joint author. III. Title.
SF201.S2 1978 636.2'08'245 77-13598
ISBN 0-7167-0025-5

Printed in the United States of America

9 8 7 6 5 4 3 2 1

16

Extenders and Extension of Unfrozen Semen

16-1 INTRODUCTION

The concentration of spermatozoa in normal, freshly ejaculated bull semen is high. Considerable extension* is necessary to provide a convenient volume to inseminate that will contain enough sperm cells to ensure maximum fertilization rates without wasting spermatozoa. Mixing spermatozoa with extender allows the addition of many ingredients that sustain and protect the spermatozoa, thereby preserving fertility until they are used for insemination. Thus the two major functions of semen extenders are to preserve the fertility of sperm cells and to increase the total volume so that the proper dose of cells for insemination can be conveniently packaged and used. A single ejaculate can therefore be used to inseminate several thousand cows.

Spermatozoa must be alive to be fertile and motility has been used to monitor viability. Motility, however, is primarily a reflection of flagellar activity, and it does not guarantee that such cells are fertile. The integrity of the genetic material in the sperm chromatin must also be preserved in order for normal embryo development to take place after fertilization. Extending media and storage conditions should preserve both functions but there is no test of semen quality prior to use that can predict how well the genetic material is maintained.

There are countless recipes for media that will preserve bull spermatozoa, several of which are described in subsequent sections. Much of the early work, particularly in Russia, has been reviewed by Anderson (1). Physiological salt solutions were found to be unsuitable for preserving sperm cells. Glucose, sulfate, and tartrate were often included in the Russian formulations, and Milovanov and Selivanova (2) reported that lecithin and peptone protected the lipid membranes of spermatozoa. None of the media, however, appreciably extended the fertile storage life of bull spermatozoa.

*"Extension" and "extender" are considered preferable to "dilution" and "diluter," since the material added to semen actively extends the life of the sperm cells.

A major breakthrough in semen extenders came when Phillips (3) reported the value of hen's egg yolk. In the original formula the extender contained equal volumes of egg yolk and phosphate buffer. Salisbury et al. (4) showed that, by replacing the phosphate with sodium citrate, equal fertility, higher spermatozoan motility during prolonged storage, and improved visibility resulted. The latter of course made microscopic evaluation easier. The yolk-citrate extender, with various modifications, has been the most widely used medium for artificial insemination in cattle. A variety of extenders have been formulated for unfrozen spermatozoa (5).

Proper identification of the bull that produced each sample of semen is extremely important. Hence all containers used from semen collection to insemination must be properly labeled. Harmless vegetable dyes are added to the extender (6, 7) to facilitate the identification of breeds, or bulls where the number is limited. Different-colored straws are used for the same purpose with frozen semen. Color seems to be a convenient aid, but no data are available on its effectiveness in reducing identification errors.

16-2 PRINCIPLES OF SPERM PRESERVATION

16-2.1 Temperature. The storage time required for practical use of most semen in artificial breeding varies from a few hours to several weeks. Metabolic rate tends to be proportional to absolute temperature, so reduced temperature has been the chief means of slowing down chemical reaction and prolonging life. Spermatozoa can tolerate temperatures somewhat higher than body temperature for short periods of time, but energy requirements are high and toxic products can accumulate rapidly. Chemical inhibition of spermatozoa is helpful at ambient temperatures (see Section 16-4).

Most of the inseminations before the advent of frozen semen were performed with semen stored at about 5°C, but upon rewarming to 37°C the percentage of progressively motile cells was observed to have decreased during storage. Metabolism continues at a reduced rate at 5°C and fertility begins to decrease before day 4 (8, 9). When semen is frozen (see Chapter 17) spermatozoa may remain fertile for years, although certain characteristics of aging are evident in semen stored at −196°C (8, 10).

At the same time that temperature is decreased, changes occur in the internal and external environments of spermatozoa. The solubility of gases in the external environment increases. For example, when there is a decline in temperature from body temperature to 0°C, solubility of O_2 doubles and CO_2 triples. With air as the gas overlying the spermatozoa, this shift in solubility of gases at lower temperatures permits a relatively greater proportion of the total metabolic activity to be due to oxidative metabolism. At higher-than-body temperatures the solubility of oxygen is relatively low in relation to the rapid metabolic rate and may become self limiting under some circumstances, even at body temperature. At low temperatures the solubility of oxygen increases,

and the metabolic activity decreases several-fold, resulting in a relatively greater oxygen supply. The solubility of certain solutes decreases, and the physical form of the colloids in the semen can change as well, causing increased semen viscosity and therefore a greater physical resistance to motility.

As temperature decreases, spatial arrangements of the internal structure and outer covering of the cells may change, a contraction of protoplasm may occur and can happen at different rates depending on the chemical components of the various structures. All such factors, as well as the rates of chemical reactions, are involved in controlling the fertile life of sperm cells by temperature reduction.

16-2.2 Energy Source. The most obvious energy requirement of the sperm cell is for motility, but additional energy is presumably required for cell maintenance. Spermatozoa are capable of both aerobic and anaerobic metabolism. A simple source of energy such as glucose or fructose should be supplied in the extender to protect intracellular reserves and cell components. Fructose, present in the seminal plasma, will be diluted considerably by semen extension. Egg yolk contains some glucose and other compounds utilizable by bull spermatozoa.

16-2.3 Osmotic Pressure and Electrolyte Balance. Spermatozoa behave as osmometers and are capable of extensive changes in size depending upon the tonicity of the medium in which they exist (11, 12). Seminal plasma has an osmotic pressure of about 285 milliosmols and it is usually assumed that this represents the ideal physiological osmotic pressure. Even if this is true, however, it should not be assumed that solutions of equivalent osmotic pressure, as measured by physical tests, are isotonic with bull spermatozoa. The permeability of the cell to different substances varies. Interactions may occur between the total concentration of substances in the fluid surrounding spermatozoa, the pH of the medium, and the specific ions and nonelectrolytes present (13, 14). If the tonicity of the medium deviates considerably spermatozoa may have bent tails, swim in circles, and die. Fortunately spermatozoa are able to tolerate a moderate range of osmolarities without a reduction in fertility (15); the degree of tolerance is affected by interactions with pH and other ions present (13). Extenders developed to preserve sperm should ideally be isotonic under the conditions used, and thus should not cause morphological or functional damage.

Solutions that are similar in composition to body fluids or are naturally produced liquids, such as egg yolk and milk, appear to be compatible with sperm. Buffered egg yolk first developed for use with bull sperm in artificial insemination was hypertonic (16, 17). Subsequently the concentrations of the salt solutions used were reduced to isosmotic levels. More recently a series of zwitter ion buffers has been used (15, 18–22) and bull spermatozoa suspended in them appear to tolerate a range of osmolarities.

16-2.4 Buffering and pH. Spermatozoa need protection from autotoxication due to acid products of metabolism, particularly when they are stored without refrigeration. Bull sperm motility and fertility are well preserved in egg yolk and milk extenders near neutral pH, although reduction of the pH to 6.5 may even be beneficial (23). The optimum pH probably varies with storage temperature and other components of the extender (13, 14). Saturation of bicarbonate media with CO_2 causes metabolic inhibition and reduces the pH to about 6.3 (24, 25, 26). This inhibition may be due in part to a reduction in pH (23).

16-2.5 Proper Gas Phase. To maintain the desired gas conditions in the extending medium, the relative proportion of gas to liquid phases in sealed containers and the composition of the gas phase should be controlled (25, 27–30). As previously stated, CO_2 inhibits spermatozoan metabolism, the degree of inhibition of anaerobic glycolysis being partially dependent upon CO_2 levels and sulfite present. Oxygen with suitable substrate can provide the sperm cells with an efficient energy source. However, toxic products such as H_2O_2 may be formed unless catalase is included (31). The effects of light are especially damaging in the presence of oxygen (31, 32, 33), and gassing with nitrogen is beneficial.

16-2.6 Inhibition of Microorganism Growth. Egg yolk and other extending media for bull spermatozoa provide a good environment for the growth of microorganisms, which produce products that may be harmful to spermatozoa, and which may infect the cow. Some organisms usually will be found in semen from healthy bulls even under aseptic semen collection and processing conditions. It is therefore standard practice to include antibiotics, such as penicillin and streptomycin, in all extenders used commercially (34, 35, 36). The various agents used, and their application, are discussed in Section 16-5.2.

16-2.7 Exclusion of Toxic Materials. Not only should extenders be formulated that will prevent the formation of toxic products during storage, but the extending media, as prepared, should be free of substances that are harmful to spermatozoa. Heavy metals, for example, are harmful to spermatozoa, but proper distillation of water insures that only nontoxic trace amounts remain. Only pure reagents should be used in formulating buffer (37). Unfortunately, cryoprotective agents such as glycerol (38, 39), required for maximum protection of spermatozoa during freezing, are somewhat toxic; and dead spermatozoa are a source of amino acid oxidase, which is harmful to surviving cells through the production of H_2O_2 (40).

16-2.8 Semen Extension and the "Dilution" Effect. Bull semen should be mixed with enough extender so that the initial sperm concentration will be

diluted to yield an optimum number of spermatozoa for insemination in a convenient volume. Studies have shown that extensive "dilution" of the spermatozoa with simple solutions depresses motility (41) but that the addition of amino acids and macromolecules such as egg albumin or casein minimizes this effect. Rottensten et al. (42) found that heated plasma mixed in equal volumes with a citrate-glucose solution improved the survival of sperm diluted up to 1:200 and stored at 4°C. Adding 25 percent egg yolk was as beneficial as the seminal plasma. Chemical inhibition can prolong motility of bull spermatozoa highly diluted with synthetic media (43). Bovine cervical mucus also counteracts the dilution effect (44).

In the experiments of Rottensten et al. (42), egg yolk and milk in practical extenders appear almost to prevent the dilution effect. Catalase, CO_2, and N_2 (29, 45, 46) may all help to eliminate the dilution effect.

The procedure used to yield suitable doses of spermatozoa for insemination is described in Section 16-7.

16-2.9 Protection of Spermatozoa Against the Cold. When mammalian sperm cells are cooled to 5°C they are subject to cold shock, which causes leakage of intracellular enzymes, potassium, lipoprotein, ATP, and other materials from the cells. Furthermore, motility is decreased and the flagellum may become bent (41). The precise mechanisms of damage are unknown, but presumably changes occur at unequal rates on the surface and internal portions of spermatozoa during cooling, and both physical and chemical damage result. Ethylenediamine tetracetic acid (EDTA) may reduce cold shock (47); and if cells are cooled slowly, particularly below 20°C (48), damage is less severe, which indicates that spermatozoa can make adaptive changes.

However, the most effective means of protecting spermatozoa against the effects of chilling is to provide them with lecithin, proteins, lipoproteins, and similar complexes found in egg yolk or milk (49–54). The importance of adding egg yolk before cooling is clearly demonstrated by experiments that show a marked beneficial effect on subsequent motility and fertility (55).

The optimum cooling rate, with regard to fertility, for semen in different extenders has not been adequately established. Extremely rapid cooling should be avoided, but otherwise considerable latitude between 1 and 4 hours is possible. There seems to be little interaction between osmotic pressure of the extenders and optimum cooling rate (56).

16-3 EXTENDERS FOR REFRIGERATED SEMEN

16-3.1 Historical. The first media used to dilute semen were primarily salt or sugar solutions designed to increase the volume of semen for immediate use rather than to conserve it (1, 41). When the value of egg yolk was discovered (3) a "preserving" extender was developed that could be coupled with the advantages of reducing metabolic activity of spermatozoa and prolonging their fertility by storage at approximately 5°C.

Semen extenders should be formulated that embody the principles of sperm preservation discussed previously. Furthermore, they should be easy to prepare, stable, and economical. Many extenders for liquid semen have been described and their development reviewed (1, 5, 20, 57, 58).

16-3.2a Egg Yolk Phosphate. In 1939 Phillips (3) first reported that fresh egg yolk combined with a phosphate buffer preserved the motility and fertility of refrigerated bull spermatozoa. The formula was: KH_2PO_4, 0.2 gram; $Na_2HPO_4 \cdot 12H_2O$, 2.0 grams; glass-distilled water, 100 milliliters; hen's egg yolk, 100 milliliters. The final pH of this medium was 6.75. In field tests this extender gave good fertility (59).

16-3.2b Egg Yolk Citrate. In 1941 Salisbury et al. (4) reported that sodium citrate was beneficial to sperm liveability when it was added to an equal volume of egg yolk. Sodium citrate had the advantage over phosphate buffer in being a chelating agent and in dispersing the fat globules in egg yolk, thereby improving visibility when spermatozoa were examined microscopically. The fertility of bull semen in phosphate and citrate extenders appeared to be equal, as indicated by the following results:

Extension rate	*Variable*	*Yolk-phosphate*	*Yolk-citrate*	*Reference*
1:4	Services/conceptions	1.53	1.53	(4)
1:200	60-90-day % nonreturns (NR)	50.5	50.5	(60)

In these studies either 3.6 percent or 3.92 percent (w/v) of sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) was used. This buffer concentration is hypertonic for bull spermatozoa but apparently, when combined with 50 percent egg yolk, did not cause osmotic problems. Later (16) the recommended concentration of sodium citrate dihydrate was reduced to 2.9 percent (w/v).

The citrate-yolk extender especially has been modified many times with varying concentrations of sodium citrate and egg yolk and the addition of glucose, sulfanilamide, and antibiotics (61). When sulfanilamide was included in the yolk-citrate extender a highly significant increase in fertility level—about five percentage units—was obtained (60, 62). This probably explains why van Dieten (63) obtained 54.6 percent NR (nonreturn) with a yolk-citrate extender containing sulfanilamide and only 50.8 percent NR ($P < 0.01$) with the phosphate extender in an experiment involving 7,101 inseminations. Also, egg yolk was reduced to 33 percent and thus the hyposmotic condition of the phosphate extender may have been deleterious (64). A nonreturn rate of 76.3 percent following insemination of 14,605 cows with sperm in a yolk-citrate-glucose extender was obtained, compared to 74.7 percent for 14,056 cows inseminated

with a yolk-phosphate extender (65). Foote and Bratton (66) also found yolk-citrate-sulfanilamide extender to be superior to yolk-phosphate. One trial (67) resulted in higher fertility for a yolk-phosphate extender than for yolk-citrate. When sulfanilamide and antibiotics were added the reverse was true, possibly because of an unfavorable antagonism between phosphate and streptomycin.

In general, yolk-citrate has been the standard against which new extenders have been compared. Salisbury and VanDemark (213) reported that glucose added to yolk-citrate increased the liveability of spermatozoa. Ohms and Willett (68) modified the 2.9 percent citrate-yolk extender by replacing part of the citrate with glucose. After one day of storage at 5°C the yolk-citrate-glucose had a 1.8 percent advantage in nonreturn rate ($P > 0.05$) and after two days of storage it had a 6.0 percent advantage ($P < 0.01$). The authors attribute the increase to the addition of glucose. Simultaneously the 50 percent egg yolk level of the control was reduced to about 17 percent egg yolk by volume in the yolk-citrate-glucose extender, and this change may also have contributed to the fertility level observed. Over a period of years the level of egg yolk used in the citrate-buffered extender devised by Salisbury et al. (4) has gradually been reduced to 20 to 25 percent by volume, and glucose or fructose is sometimes added.

16-3.2c Ratio of Yolk to Buffer. The early work was done with equal volumes of egg yolk and buffer. Using smaller amounts of egg yolk often yielded inferior results, probably because of the mitigating effect of the egg yolk on the hyperosmotic citrate solutions used and on the high concentrations of phosphate ions in the phosphate extender.

Table 16-1 shows a summary of fertility results, which clearly indicate that there was generally no fertility advantage in reducing the proportion of egg yolk and simultaneously increasing the sodium citrate but that, for convenience, the proportion could be reduced considerably. With the development of more complete buffers to combine with egg yolk, however, a reduction in the volume of egg yolk from 50 to 20 percent improved sperm survival with many buffer combinations tested (66). Fertility was also improved significantly over the standard 50 percent yolk-citrate extender (see Table 16-1).

Under most conditions where osmolarity, pH, and other characteristics of the buffer alone appear to be optimal for sperm survival, egg yolk level may be reduced from 20 to 1 percent. This is particularly true at ambient temperatures (see Section 16-4). Table 16-2 shows an example of the importance of the egg yolk level as it interacts with four other factors during an 8-day storage period. The effects of extender, molarity, and yolk level were large ($P < 0.005$), accounting for a majority of the non-bull variance. Temperature had no effect, showing that with proper media bull spermatozoa can survive for 8 days at 25°C as well as at 5°C. Also pH had no effect. By far the largest interactions were extender × temperature (CUE—Cornell University Extender—was best

Table 16-1. Fe
proportion of eg

		% egg
50	*33*	*25*
67.1		66.0
66.7		63.9
70.8		69.2
73.5		
66.2		
65.8		
63.1		
		74.0
73.4[a]		

[a]16-week nonreturns.
[b]6-month nonreturns.
[c]120–150-day nonretur
[d]12-week nonreturns.
[e]$P \leq 0.01$ differences

at 25°C and Tris was best
egg yolk was clearly best
large and significant int
× molarity, and molarity

16-3.2d Complex Citrat
developed complex buffe
plex buffers, and they rep
They also reported on the
room temperature, but it
well. Table 16-3 shows th
CU-16, and of two modifi
(77). Modifications of the
0.3 to 1.2 percent and the
these and similar extende
gassed with CO_2. The lat
that two modified IVT ex
age 60–90-day nonreturn
yolk–skim-milk control.

Table 16-1. Fertility results (percent nonreturns) as the proportion of egg yolk to citrate is decreased.

% egg yolk						*Total insemi-*	
50	*33*	*25*	*20*	*17*	*12.5*	*nations*	*Ref.*
67.1		66.0				3,172	(69)[a]
66.7		63.9				2,929	(70)[b]
70.8		69.2				2,591	(71)[c]
73.5			72.3			11,013	(72)[d]
66.2			69.5			5,908	(73)
65.8				63.4		2,809	(70)[b]
63.1					62.4	2,484	(70)[b]
		74.0[e]		66.2[e]		2,713	(71)[c]
73.4[e]			76.3[e]			18,494	(74)[e]

[a]16-week nonreturns.

[b]6-month nonreturns.

[c]120–150-day nonreturns.

[d]12-week nonreturns.

[e]$P \leq 0.01$ differences due to chance.

at 25°C and Tris was best at 5°C), and temperature × level of yolk (20 percent egg yolk was clearly best at 5°C and 5 percent yolk was best at 26°C). Other large and significant interactions were extender × molarity, temperature × molarity, and molarity × egg yolk level.

16-3.2d Complex Citrate-Buffered Yolk Extenders. Foote et al. (74, 75) developed complex buffered yolk extenders based on previous work with complex buffers, and they reported beneficial effects of glycine (76) and CO_2 (24). They also reported on the use of Illini Variable Temperature (IVT) extender at room temperature, but it has been used as a bull-semen extender at 5°C as well. Table 16-3 shows the original composition of this extender, of CUE and CU-16, and of two modifications called 14 and 14G developed in New Zealand (77). Modifications of the IVT have been made by increasing the glucose from 0.3 to 1.2 percent and the egg yolk from 10 to 20 percent. The composition of these and similar extenders is given by Bartlett and VanDemark (25). All are gassed with CO_2. The latter workers, in a limited test totaling 765 cows, found that two modified IVT extenders containing 20 percent egg yolk yielded average 60–90-day nonreturns over a 5-day use period comparable to a citrate-yolk–skim-milk control.

An extensive test (78) of a similar CO_2-gassed extender with glycine and glutathione added gave results as follows:

Age of semen (days)	*CO_2 extender*		*Yolk-citrate*	
	Cows bred	*% NR*	*Cows bred*	*% NR*
1	724	78	279	74
2	2,293	76	646	68
3	2,272	72	506	62
4	2,005	66	50	60
5-7	1,363	64	—	—

Melrose (79) obtained a 56 percent 112-day NR rate for bull sperm in IVT used at 5°C over a period of 2 to 6 days. A 9 percent skim-milk powder-glycerol control averaged only 40 percent over this period. It is clear from these examples that semen in IVT can be used successfully for several days at 5°C. One disadvantage has been the variability in results achieved, which presumably reflects varying degrees of maintaining the proper CO_2 level following gassing (26).

The CUE extender (74, 75) was developed to maintain proper pH and to generate CO_2 through the use of bicarbonate-citric acid buffer system. This was compared with a simpler extender, CU-16 (see Table 16-3). When bull semen was extended to 10 million motile spermatozoa per milliliter and stored for 12 days at 5°C, sperm survival in CUE and CU-16 was significantly better than in the yolk-citrate control. A field trial with semen processed as in the motility trial, but with most semen used for insemination within three days of collection, gave the following results (75):

Extender	*No. 1st inseminations*	*% 60–90-day NR*
2.9% citrate (EYC)	6,069	73.4
CUE	6,280	76.6
CU-16	6,145	76.0

When put into routine use in the field 290,053 inseminations averaged 74.9 percent 60-90-day NR, an increase of two percentage units over a comparable period when the 2.9 percent citrate-yolk extender was used. Singleton and Josey (80) also reported that CUE and CU-16 were superior to EYC for bull sperm at 5°C.

Table 16-2. Sperm motility as affected by storage temperature, buffer type, osmolarity, pH, and egg yolk levels. Means of fifteen ejaculates after storage for eight days.[a]

Buffer concentration	*pH*	*Stored at 5°C*				*Stored at ambient temperature (25°C)*			
		% egg yolk				*% egg yolk*			
		0	*1*	*5*	*20*	*0*	*1*	*5*	*20*
		(CUE extender)							
50%[b]	6.50	11	27	27	43	20	20	31	38
	6.75	17	33	28	39	17	29	28	43
75%	6.50	1	54	49	51	50	57	48	45
	6.75	0	53	49	58	31	54	51	43
100%	6.50	0	47	46	46	42	55	55	41
	6.75	0	47	47	49	33	59	54	50
		(Tris-yolk extender)							
0.15M	6.50	5	30	37	49	13	13	14	14
	6.75	3	31	30	50	7	12	5	15
0.20M	6.50	3	34	41	44	18	15	29	33
	6.75	2	27	43	53	15	17	25	23
0.25M	6.50	0	28	31	29	16	23	34	27
	6.75	0	35	43	43	13	25	31	16

[a]Foote, R. H. Unpublished data. Cornell University. 1960.

[b]Buffer concentration as a percentage of strength shown in Table 16-3.

Shannon (77) reported on extensive field testing of bull semen (see Table 16-4) with several of the extenders listed in Table 16-3. All extenders contained 20 percent egg yolk by volume. With the short breeding season in New Zealand, fertility is calculated on the basis of 49-day NR to insemination. The fertility results were significantly lower in the control yolk-citrate extender, which confirmed previous studies (75). Particularly striking was the difference in fertility after two days' storage. It is believed that the ability of the CUE and CUE-G extenders to maintain high fertility over a period of several days is due to the CO_2 liberated from the sodium bicarbonate. However, part of the beneficial effect observed previously with extenders 14, 14G, and CU-16 appears to result from an improvement in the nonelectrolyte balance through the addition of glucose and glycine.

Table 16-3. Composition of buffered yolk extenders.[a]

Component	Citrate	g/100 ml of distilled water				
		IVT[b]	CUE	CU-16	14	14G
Sodium citrate dihydrate	2.9	2.00	1.45	1.45	2.00	2.00
Sodium bicarbonate	—	.21	.21	—	—	—
Potassium chloride	—	.04	.04	—	—	—
Citric acid monohydrate	—	—	.09	—	—	—
Glucose	—	.30	.30	1.25	.30	.30
Glycine	—	—	.94	.94	1.00	1.00
Glycerol	—	—	—	—	—	1.25
Sulfanilamide	0.6	.30	.30	.30	—	—

[a]Final extenders consisted of 20 parts egg yolk and 80 parts buffer to which antibiotics were added. In some studies 50% egg yolk was combined with the citrate. Adapted from references 24, 74, 75, and 77.

[b]Gassed with CO_2.

Table 16-4. Fertility of bull spermatozoa stored at 5°C in yolk extenders, based on 49-day nonreturns (NR)[a].

Extender[b]	Age of semen when used					
	Day of collection		Stored 1 day		Stored 2 days	
	No. inseminations	% NR	No. inseminations	% NR	No. inseminations	% NR
CUE	59,091	62.8	25,309	65.2	1,190	62.4
CUE-G	30,058	63.1	13,909	64.9	2,392	62.9
14	73,993	62.8	37,758	63.5	2,681	59.2
14G	35,351	62.7	17,597	64.9	932	60.0
Citrate	98,605	60.6[b]	44,610	60.8[c]	2,788	51.5[c]

[a]From Shannon. *New Zealand J. Agric. Res.* 7:357–363. 1964.

[b]See Table 16-3 for composition. CUE-G was CUE plus 1.25% glycerol (w/v). All extenders contained 20% egg yolk by volume.

[c]Significantly different from all others, $P < 0.01$.

The CUE formu
without appreciably
ifications follow.

Extender
CUE
CUE + catalase, 2
CUE
CUE + catalase, 2
CUE
CUE + catalase, 1
CUE, pH 6.80 (co
CUE, pH 6.65
CUE, pH 6.50
CUE, 300 mosmol
CUE, 333 mosmo

[a]None of the differe

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The CUE formula has been modified slightly by different workers but without appreciably changing fertility results. Several examples of these modifications follow.

Extender	*No. 1st inseminations*	*% 60–90-day NR*[a]	*Reference*
CUE	6,913	71.0	(45)
CUE + catalase, 20 μg/ml	7,615	71.5	
CUE	10,309	76.1	(45)
CUE + catalase, 20 μg/ml	10,951	74.6	
CUE	6,064	72.9	(81)
CUE + catalase, 1 μg/ml	6,243	73.2	
CUE, pH 6.80 (control)	23,727	74.5	(23)
CUE, pH 6.65	24,779	74.7	
CUE, pH 6.50	22,577	74.7	
CUE, 300 mosmols	12,797	74.0	(15)
CUE, 333 mosmols	13,966	74.0	

[a]None of the differences was statistically significant, $P > 0.05$.

Catalase has been shown to protect sperm under conditions that presumably lead to hydrogen peroxide formation (33, 41). The small amount of catalase in egg yolk, however, is apparently sufficient to protect sperm under the usual dark storage conditions at 5°C, where added catalase has only minor effects.

The pH of CUE extender may be decreased to at least 6.5 without harmful effect by increasing the citric acid component of the buffer. Other experiments with CUE at the lower pH have given actual fertility values higher than for control CUE. Pickett et al. (82) recommended the lower pH range and obtained poorer fertility with CUE as the pH approached 7.0.

Other studies (15) indicate that minor changes in osmotic pressure are not critical. These and numerous other trials with CUE or modifications (83–86) have yielded high fertility results with the extenders used over a period of a few days at 5°C.

New Zealand researchers (87) improved the citrate-buffered yolk (see Table 16-3) by gassing with nitrogen and adding caproic acid. This led to an extender called "Caprogen." Caprogen consists of 2.0 percent sodium citrate dihydrate, 0.3 percent glucose, 1.0 percent glycine, 1.25 percent glycerol, 0.0325 percent caproic acid, and 0.01 percent sulfacetamide, to which is added 20 percent egg yolk, penicillin, and streptomycin. In 1961 (88) the following fertility results were reported.

Age of semen	*14G*		*14G + caproic acid*	
	No. inseminations	*% 49-day NR*	*No. inseminations*	*% 49-day NR*
Day of collection	224,000	64.1	42,000	63.7
Day after collection	96,000	64.3	17,000	66.9

The addition of caproic acid increased NR rates on semen stored for a day at 5°C ($P < 0.01$). A subsequent trial was initiated comparing caproic acid additions and nitrogen gassing. These results are summarized in Table 16-5. The caproic acid and/or gassing with nitrogen improved fertility of semen used after the first day. Thereafter Caprogen, called 14GCN, was used routinely in New Zealand for extending sperm stored at 5°C. Later (see Section 16-4) this was modified for ambient temperature use by reducing the egg yolk content. Balasov et al. (89) also reported that caproic acid was beneficial when added to a yolk-citrate-glucose extender.

Following the work of Roy and Bishop (76) there were numerous reports that yolk-glycine and yolk-glycine-glucose improved sperm survival (5, 57). However, fertility results were often disappointing. For example, Graham and Erickson (90) used a yolk-glucose-glycine extender to inseminate 3,982 cows and obtained 65.8 and 35.7 percent 60–90-day NR on the day of collection and the day after collection. These results were 7.6 and 24 percent below the heated

Table 16-5. Fertility (% 49-day nonreturns) of bull spermatozoa stored at 5°C in extenders gassed with nitrogen and/or containing caproic acid.[a]

	Age of semen when used					
	Day of collection		*Stored 1 day*		*Stored 2 days*	
Extender[b]	*No. inseminations*	*% NR*	*No. inseminations*	*% NR*	*No. inseminations*	*% NR*
14G (Control)	101,982	65.2	56,687	65.4[c]	2,061	62.3
14GC	99,420	65.6	52,064	66.7[d]	5,854	63.5
14GN	50,637	65.8	27,163	66.2[d]	2,359	61.9
14GCN	52,436	65.8	35,435	67.6[d]	739	64.8

[a]From New Zealand Dairy Board. 48th Farm Production Rpt. 1971–1972.

[b]C = caproic acid; N = nitrogen gassing; CN combination is called Caprogen.

[c,d]Numbers with different superscripts in the same column, $P < 0.01$.

whole milk control used simultaneously to inseminate 3,722 cows. Consequently, glycine has been little used recently, except in more complex extenders such as CUE or Caprogen.

A variety of buffered yolk preparations have been reported in the Russian literature, but the exact composition is often not given. These reports have been summarized by Turton (91). One extender extensively tested with regard to fertility was a yolk-sodium-citrate-saccharose-sodium-bicarbonate-potassium-phosphate extender. When semen was stored for up to three days at 5 to 15°C, 74.3 percent of 5,998 cows inseminated conceived. Semen stored at 0°C in a yolk-citrate-glucose control extender and used to inseminate 6,231 cows resulted in 65.0 percent conception.

16-3.2e Tris-Buffered Egg Yolk Extenders The development of zwitter-ion buffers of Good (92) has led to the examination of many for buffering egg yolk as extenders for bull spermatozoa (18-22). These buffers were shown by Good et al. (92) to be relatively harmless to various cell suspensions, and they provided excellent buffering capacity over a suitable pH range. In early studies (18, 19) bull sperm survival at 5°C in Tris-buffered egg yolk was equal or superior to that found for egg yolk citrate and CUE. Optimum sperm survival was obtained in 0.2 molar Tris-yolk extender, adjusted with citric acid to pH 6.75. A nomogram for preparing Tris solutions with different osmolarity and pH characteristics has been published (14).

Among the compounds that have been tested (21) using sperm motility as a criterion of suitability, are TRIS, tris (hydroxymethyl) aminomethane; TES, N-tris (hydroxymethyl) methyl-2-aminomethanesulphonic acid; MES, 2-(N-morpholino) ethanesulphonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; PIPES, piperazine-N,N'-bis(2 ethane sulfonic acid); MOPS, 2-(N-morpholino) propane sulfonic acid; and BES, N, N bis (2-hydroxyethyl)-2 aminoethane sulfonic acid. These compounds can be combined and titrated to provide buffers ranging in pH and osmolarity that are relatively innocuous to spermatozoa (20). They can be combined with egg yolk to give suitable extenders for temperatures ranging from 25 to −196°C.

Only Tris has been extensively fertility-tested with bull spermatozoa. Extensive studies were carried out before the fertility testing to establish optimum levels of Tris in combination with egg yolk and other substances (14, 18). Glycerol was included as one component because of the observed beneficial effects of glycerol at 5°C in milk extenders and the need for such a compound in extenders for freezing semen. The results of one study (see Table 16-6) showed that glycerol could be included in the initial extender, thus avoiding the time-consuming steps of adding glycerol at 5°C, as is often done with other extenders. There was no difference in times of glycerol additions, but motility of spermatozoa was lower in media with a pH of 6.25 ($P < 0.01$) and when the molarity of the Tris was 0.25 ($P < 0.01$).

Table 16-6. Spermatozoan motility when glycerol was added directly to Tris-yolk extenders or after cooling to 5°C.[a] (Average of 20 samples stored for 8 days at 5°C.)

Glycerol added	*Molarity of Tris*	*pH*		
		6.25	*6.50*	*6.75*
		(% motile spermatozoa)		
Initially	.20	47	55	55
	.25	40	49	52
At 5°C	.20	47	55	53
	.25	42	47	51

[a]From Foote. *J. Dairy Sci.* 53:1475–1477. 1970.

The Tris extender that was selected for fertility testing (15) consisted of the following:

Tris	3.028 g
Citric acid monohydrate	1.675 g
Fructose	1.25 g
Glycerol	8.0 ml
Glass redistilled water	92.0 ml
Egg yolk (+ antibiotics)	25.0 ml

Semen was extended an average of 1:108 to give 8 million motile sperm per milliliter and the 0.2M Tris-fructose-glycerol-yolk extender, pH 6.75, was compared with CUE.

Extender	*No. 1st inseminations*	*% 60–90-day NR*
CUE	5,981	73.0
Tris	5,673	73.3

These results showed clearly that most of the ions in traditional buffers could be replaced by Tris. Other field trials established that glycerol was beneficial in the Tris extender (see Table 16-7). The difference, in favor of glycerol, was 2.5 percent ($P < 0.01$). There was no statistically significant effect of pH, although the lower pH tends consistently to be associated with higher fertility (23). Tris and CUE gave equal fertility results. Another field trial with over

40,000 inseminations resulted in 73.0 percent 60–90-day NR for CUE and 73.4 percent for Tris, thus confirming the equivalency of the two extenders.

Tris has been used successfully for storing buffalo spermatozoa (93). It has been used for titrating other zwitter-ion buffers (21). When combined with CUE (85) 4,080 inseminations resulted in 72.3 percent 60–90-day NR versus 69.7 percent for Spermasol T. When compared with Spermasol milk extender (86) the CUE-Tris was superior, declining only two percentage units in fertility over a period of four days. When semen was collected and processed only twice a week for use throughout the week, 137,074 first inseminations gave 70.2 percent 60–90-day NR. The formula for preparing one liter of the CUE-Tris extender is 7.57 grams Tris, 2.50 grams fructose, 5.53 grams citric acid, 10.88 grams sodium citrate, 1.35 grams sodium bicarbonate, 0.25 gram KCl, 2.25 grams glucose, 7.03 percent glycerol, and 2.25 grams sulfanilamide. To this is added 10 to 20 percent egg yolk and antibiotics.

It is clear that Tris types of organic buffers provide a versatile series of solutions that can be used to formulate desirable semen extenders. They are suitable for ambient temperature storage, refrigerated, or frozen semen.

16-3.2f Other Egg Extenders. A whole-egg extender was tested by Dunn et al. (94). Preparation of the extender was simple because the whole eggs with the chalazae removed were simply blended and added to buffer. The pH of a citrate/whole-egg medium was adjusted from about 7.9 to 6.7 by replacing sulfanilamide in the conventional 2.9 percent citrate-sulfanilamide-yolk medium with sulfasuxidine. Adjustment of the pH is essential for good sperm survival. The whole-egg formulation averaged 61.6 percent 60–90-day nonreturns compared to 62.3 percent for the egg yolk control. Whole-egg extenders have not been adopted commercially.

Table 16-7. Fertility of bull spermatozoa in liquid Tris extender *vs* CUE.[a]

Treatments	*Osmolarity (mosmols)*	*No. 1st services*	*% 60–90-day NR*
CUE Control	333	13,966	74.0
Tris, pH 6.5	323	14,376	71.9
Tris, pH 6.75	317	13,933	71.2
Tris-gly, pH 6.5	–	13,630	74.0
Tris-gly, pH 6.75	–	12,903	73.1

[a]From Foote. *J. Dairy Sci.* 53:1475–1477. 1970.

Various prepared extenders incorporating egg yolk have been marketed commercially (5, 41, 95). In Japan Neoseminan is an ampuled sterilized product reported to be stabile for several years. It consists of egg yolk, sodium citrate, potassium citrate, sodium phosphate, glucose, homosulfamine, sodium sulfamesadin, and chlorpromazine and is gassed with CO_2. Spermasol (see Section 16-3.3e) has been used principally in Germany. Many formulations containing sodium citrate, potassium phosphate, and gelatin have been combined with either egg yolk or milk. Other extender formulations have been marketed as well (60). Although they offer convenience, any of the complete formulations containing egg yolk or similar organic material probably have limited shelf life. Large AI organizations therefore prepare their own extenders shortly before use.

16-3.3a Milk—History and Heating. Phillips and Lardy (96) observed that evaporated milk could maintain sperm for only 48 hours. Fresh semen gave higher fertility than semen extended and stored in yolk-phosphate or in autoclaved milk (97). Better results were obtained by Michajilov (98), who used boiled filtered milk for jack semen. A canned sterile skim milk was easy to prepare, sperm were clearly visible, and fertility was superior to that allowed by the yolk-citrate used previously (99). Later Jacquet and Cassou (100) found that the quality of the canned milk product varied and they recommended that 10 percent by weight of powdered milk plus antibiotics be added to double-distilled water and that egg yolk also be included up to 10 percent of the final volume.

Milk preparations were being investigated simultaneously by Almquist and associates, and details of proper milk handling as a bull semen extender were described (101). Milk must be heated beyond the pasteurization point to destroy lactenin, a spermicide (102). Sulfhydryl groups released by heating probably inactivate the toxic factor. Recommended procedure is to heat milk in a double boiler to 92 to 95°C for 10 minutes, cool it to 5°C, and add antibiotics. The extender is then ready for use. Other methods of inhibiting the toxic factor have been reviewed (5, 41); they include the use of thioglycolic acid or cysteine. Both contain sulfhydryl groups. Jones (103) postulated that excess cysteine might be harmful and later reported that dialysis to remove excess cysteine was beneficial (104). Heating, however, was preferable. Many laboratories in the 1950s observed that egg yolk also made heating the milk unnecessary and this was documented in the literature (105).

Fortified skim milk was found, as a semen extender, to be detrimental to spermatozoa (106). Different batches of skim milk also required different degrees of heating to inactivate the toxic factor (107). Too much heat can be harmful, possibly because of its effect on lactose (103). Others have observed variability in batches of liquid milk and dried milk. This and the different procedures used in heat treatment of milk may be responsible for some of the

variability in the fertility results that investigators have obtained in comparing milk and egg yolk extenders.

16-3.3b Liquid and Dried Milk Preparations. Liquid whole milk, skim milk, cream, and various reconstituted powdered milks have been tested as bull semen extenders. The early literature has been reviewed (5, 41). Since sperm motility is difficult to assess in many milk preparations only the fertility results of studies are meaningful.

Table 16-8 summarizes fertility tests run on several milk extenders in which antibiotics were used. Most of the fertility results were based on 60–90-day nonreturns to service, but if fertility was based on other time intervals the comparisons within an experiment were made on the same basis. The first series of experiments all included egg yolk-citrate as a control. In general egg yolk-citrate and milk extenders gave comparable results. Most differences were small and appeared to be due to chance. Variations in the levels of egg yolk (20 to 50 percent), citrate (2.9 to 3.6 percent), and antibiotics, as well as the formulation and batches of milk used, also presumably contribute to the variations observed. Saacke et al. (106) called attention to the importance of milk solids and osmolarity: Although milk proteins can protect spermatozoa against moderate shifts in osmolarity (104) failure to observe the principles of sperm preservation discussed earlier in this chapter can reduce fertility.

In the studies by Almquist (109) and Melrose et al. (114) semen from bulls exhibiting low fertility appeared to benefit from being stored in milk. It is possible that the antibiotics were somehow more effective in the milk preparation as processed. Morgan et al. (119) showed that *Vibrio fetus* infection was controlled by two hours' exposure at 5°C to streptomycin in skim milk but not in egg yolk. At the time of these studies vibriosis was widespread in cattle and had not yet been eradicated from most bull studs.

Homogenized milk, homogenized cream (9 percent fat), and skim milk are capable of yielding comparable fertility results. Skim milk, however, allows spermatozoa to be seen microscopically and sperm viability can therefore easily be assessed. Moreover, skim milk powder is readily available and inexpensive. Large batches can be pretested and, if found to be satisfactory, can be used with confidence in their uniformity. The sterilized cream (115) is convenient to use because it requires no additional heating, and large batches can be secured for use over a considerable period of time. Reconstituted buttermilk (not shown in Table 16-8) can also be used (120). Spermatozoa extended in reconstituted buttermilk (10.8 grams with 89.2 milliliters of distilled water plus antibiotics) gave good results in a limited fertility test when compared with yolk-citrate extended semen.

16-3.3c Milk Combined with Sugar, Glycine, and Glycerol. Although the fertility of bull spermatozoa extended in milk was initially good, it often declined rapidly with storage at 5°C. This was also true of the original yolk-citrate

Table 16-8. Fertility of bull spermatozoa in heated fresh or powdered milk with sugars, glycine, or glycerol.

Treatment[a]	*No. 1st inseminations*	*% NR*	*Reference*
Homogenized milk	2,931	59.9[b]	(73)
Homogenized milk-sulfanilamide	4,375	64.6	
Yolk-citrate-sulfanilamide	2,892	69.5	
Homogenized milk	1,570	76.1	(108)
Homogenized milk + cornstarch	1,554	74.3	
Yolk-citrate	1,565	75.9	
Homogenized milk + cornstarch	2,852	65.3	(108)
Skim milk + cornstarch	2,873	67.8	
Yolk-citrate-sulfanilamide	2,674	66.8	
Homogenized milk	4,178	71.6	(109)
Yolk-citrate	4,222	64.2[b]	
Homogenized milk	10,012	69.4	(110)
Yolk-citrate	9,927	71.2	
Homogenized milk	4,637	68.8	(111)
Yolk-citrate	5,051	69.9	
Skim milk	6,850	70.7	
Yolk-citrate	7,979	69.1	
Homogenized or skim milk	23,422	69.8	(112)
Yolk-citrate-sulfanilamide	24,269	69.6	
Skim milk	44,746	69.0	(113)
Skim milk powder, 10% w/v	48,013	69.3	
Skim milk powder, 9% w/v	5,076	68.4	(114)
Yolk-citrate	5,113	63.5[b]	
Skim milk powder, 9% w/v	2,276	65.7	(114)
Skim milk powder + 12.5% yolk	2,339	68.1	
Skim milk powder, 9% w/v	1,521	66.3	(114)
Skim milk powder + 3% glycine	1,421	67.2	
Skim milk powder, 9% w/v	2,025	67.3	(114)
Skim milk (liquid)	1,949	68.9	
Skim milk	2,263	69.2	(115)
Sterilized homogenized cream	2,931	69.4	
Yolk-citrate	2,370	63.6	
Homogenized milk	25,599	71.3	(116)
Homogenized milk + 1.25% fructose	25,282	72.4	
Skim milk	14,319	61.5	(117)
Skim milk + 10% glycerol		64.5	
Skim or homogenized milk	10,845	63.7	(118)
Skim or homogenized milk + 10% glycerol	10,831	71.5[b]	

[a]All extenders contained antibiotics (streptomycin and often penicillin) and all liquid milks were heated excepting when egg yolk was added. Egg yolk content varied from 20 to 50% in the yolk-citrate controls.

[b]Values that differ significantly from others within an experiment, $P < 0.05$.

extender, and was partially responsible for the development of more complex yolk extenders created to prolong fertility of unfrozen semen. Milk is low in hexoses, although traces of glucose are present, and it contains lecithin and the fat-decomposition products glycerol and organic acids. Spermatozoa cannot utilize lactose, but they can metabolize the other substances aerobically and glucose anaerobically. No advantage was found in adding fructose to heated homogenized milk (see Table 16-8). Semen was used uniformly for four days and the decline in fertility with fructose added was 5.5 percent versus 4.6 percent for the milk extender alone (116). Thus at 5°C an additional energy source for spermatozoa appeared unnecessary.

The effect of adding glycerol to milk is usually favorable. Bratton and Foote (73) obtained, with and without glycerol, 65.8 and 64.4 percent 60–90-day NR in heated homogenized milk. Williams et al. (121) conducted a large scale experiment (14,341 inseminations) and reported that 10 percent glycerol added to heated homogenized milk increased the 60–90-day NR after 1, 2, and 3 days of storage at 4°C by 2.5, 3.5, and 4.2 percent ($P < 0.01$). Almquist (118) also showed that glycerol markedly improved the fertility of bull sperm stored for several days at 5°C. On the other hand, O'Connor and Smith (117) found that glycerol helped to maintain the fertility of bull sperm only during the last two of four days of storage at 5°C. A comparison of these results follows:

	No. 1st inseminations		*Advantage of glycerol in % NR*	
Age of semen	*O'Connor*	*Almquist*	*O'Connor*	*Almquist*
1 day	3,682	7,106	− 3.1	+ 2.3
2 days	3,494	8,707	− 1.3	+ 7.4
3 days	3,688	3,405	+ 4.7	+12.2
4 days	3,455	2,458	+10.6	+19.5

No difference in fertility was found between semen from the same source extended with heated skim milk to which either 5 or 10 percent glycerol had been added (122), although 5 percent glycerol gave the best spermatozoan liveability. Glycerol added to skim milk at room temperature reduced motility and induced morphological changes in spermatozoa. Slow addition of glycerol at 5°C was recommended. In another study (79) fertility was reduced from 64.6 to 55.6 percent by including 10 percent glycerol initially in a 9 percent skim milk powder extender mixed with sperm cells at 20°C. Overall, the studies indicate that, with the proper preparation of milk and milk-glycerol extenders, initial fertility of spermatozoa in each is similar, but is better maintained for several days at 5°C with milk-glycerol extender.

16-3.3d Milk-Egg Yolk Combinations. After early work indicated that small volumes of egg yolk added to milk might improve sperm survival and inactivate the spermicide in milk, many combinations were tested. The early work was reviewed in the previous edition of this text (123). Table 16-9 shows several combinations of milk with yolk and glycine or glycerol. All liquid milks and most reconstituted milks used were heated to 92°C for 10 minutes, and all extenders listed in the table contained antibiotics.

The addition of glycine to milk was based on the sometimes favorable results obtained with yolk-glycine extenders (see previous sections). However, it is clear that under the conditions tested glycine did not improve fertility; in fact, when it was combined with glycerol and milk-yolk (see Table 16-9) it decidedly depressed fertility. Hence glycine has not been used as a regular constituent of milk extenders.

Table 16-9. Fertility of bull spermatozoa in milk-yolk combinations.

Treatment	*No. 1st inseminations*	*% NR*	*Reference*
Homogenized milk	3,033	73.7	(124)
50% milk–25% yolk-25% of a 3% glycine solution	2,848	72.3	
Homogenized milk + 10% glycerol	4,850	71.4	(124)
Milk-yolk-glycine + 10% glycerol	4,078	60.9[b]	
Skim milk-10% yolk + 8% glycerol	2,552	73.5	(125)
Yolk-citrate + 6.32% glycerol	2,542	74.4	
Skim milk + 5% yolk[a]	4,045	67.0	(126)
Skim milk-5% yolk + 10% glycerol	4,032	67.0	
Skim milk-5% yolk	8,438	76.3	(126)
Skim milk-5% yolk + 10% glycerol	7,947	77.7	
Milk-yolk	3,665	63.4[b]	(127)
CU-16 (yolk base)	3,853	68.3	
9% skim milk powder, 12.5% yolk	11,524	63.3	(79)
CUE (complex buffered yolk)	11,339	64.6	
9% skim milk powder, 6% yolk	20,610	63.2	(128)
9% skim milk powder, 6% yolk, 1.5% glycerol	20,630	63.8	
9% skim milk powder, 6% yolk	16,983	63.3	(128)
9% skim milk powder, 10% yolk, 3% glycerol	16,983	62.6	

[a]In the first study by these authors glycerol was added at room temperature and 90–120-day % NR calculated. In the second study glycerol was added at 5°C and 30–60-day % NR calculated.

[b]$P < 0.01$ comparing fertility within experiments.

The addition of glycerol to liquid milk and egg yolk or to powdered milk with egg yolk was consistently without measurable effects. Glycerol does not appear to improve the fertility of semen stored for several days in milk-yolk extenders, in contrast with its demonstrated effect when added to milk alone (128). It is possible that egg yolk serves a similar function to that of glycerol in milk extenders, but the results of appropriate tests of that hypothesis, using the milk and milk-yolk extenders with and without glycerol, have not proved the hypothesis. It appears, however, that semen can be added directly to glycerolized milk-yolk extender (126). This could simplify processing, especially of frozen semen.

Milk-yolk extender seems comparable to CUE (79), although after 3 days of storage the 112-day nonreturn rates were 62.5 percent for CUE and 57.8 percent for milk-yolk extender. A report by Hutchinson and Cooper (129) indicated that 3 percent glycerol added to 87 parts of heated skim milk and 10 parts of egg yolk improved fertility substantially over a yolk-citrate control. A similar extender made from powdered skim milk (128) was not superior to milk-yolk when bull spermatozoa were stored for up to 3 days before insemination.

Thus the simplest satisfactory milk-yolk semen extender appears to be one made of either heated fresh or powdered skim milk (9 to 10 percent solids) with 5 to 10 percent fresh egg yolk added. Such an extender was used regularly by several AI organizations until the practice of freezing semen came into use.

16-3.3e Other Extenders. Various commercial preparations have been used successfully. Spermasol, for example, is an egg yolk-citrate-phosphate-gelatin product that can be combined with milk (130); Laiciphos is a powdered milk extender that gives good fertility when mixed with 10 percent fresh egg yolk (131). Tomato juice and other vegetable materials have been combined with yolk (41) and countless variations in recipes for semen extenders can be found in the literature (5, 41, 132). Examples of those tested extensively for ability to yield high fertility have been summarized in the tables. Certain extenders designed primarily for storage at ambient temperatures (see Section 16.4) have received limited use at 5°C.

Testing extenders for commercial insemination of cattle has served as a basis for developing extenders for other species. Similar extenders preserve buffalo sperm equally well (133, 134, 135).

16-4 EXTENDERS FOR STORAGE AT AMBIENT TEMPERATURES

16-4.1 Development of Room Temperature Extenders. It is clear from the survival rates of sperm in the epididymis and the female reproductive tract that sperm cells can remain viable at body temperature for significant periods of time. Refrigerating semen at 5°C and freezing semen have greatly increased

its *in vitro* storage life. The need for unrefrigerated semen has therefore diminished, but in some areas refrigeration is not widely available or is expensive. Hence consideration will be given to techniques of preserving bull sperm at ambient temperatures.

Storing spermatozoa in nutrient media at ambient temperatures obviously makes it necessary to control bacteria. According to Foote and Bratton, a combination of sulfanilamide, penicillin, streptomycin, and polymyxin is extremely effective in preventing bacterial growth in bull semen extended with yolk-citrate and stored at 25°C (35). Under these conditions sperm survival is greatly improved and egg yolk is stabilized. Foote and Bratton conclude that "this combination of antibacterial agents gives promise of making possible the development of an extender for bovine semen which will not require refrigeration."

16-4.2 CO_2 Ambient Temperature Extenders. Biochemical control of spermatozoan metabolism is prerequisite for a good extender that can be used at room temperature. Previous sections of this chapter have referred to the Illinois work showing that CO_2 markedly reduced sperm metabolism. When the CO_2 level was properly controlled the motile and fertile life of bull sperm was prolonged (25). A successful ambient temperature extender called IVT (see Table 16-3) was developed (24). These workers reported little change in fertility when a limited number of cows were inseminated with semen stored up to seven days at ambient temperature.

IVT has been used extensively for boar semen where storage temperatures of 15°C or higher are preferable to 5°C. It has not been widely used for bull sperm, however, due to the requirements for CO_2 gassing and for sealing the storage vial so it will hold proper CO_2 levels.

CUE, a related extender (see Section 16-3) that includes glycine and does not require external gassing with CO_2, has given consistently good sperm survival at 25°C. CU-16, a simplified extender, similarly preserves sperm motility. In each of these glycine may promote the desirable production of CO_2 as a result of sperm metabolism. Boye (136) studied temperatures of 5, 10, 15, 20, 25, and 30°C for storing bull spermatozoa in CUE and found 20°C to be the optimum. The insemination of 1,978 cows with CUE-extended semen stored for 1, 2, and 3 days gave 81, 79, and 79 percent NR on a 30–60-day basis.

Abdu (137) reported that ethylenediamine tetracetic acid (EDTA) added to a yolk-citrate-glucose medium gave good fertility when semen was stored in it at 18 to 25°C and used after 3 to 6 days. Only limited breedings were carried out.

16-4.3 Caprogen. By far the most extensive testing of an ambient temperature extender has been done on Caprogen in New Zealand. The initial development of this extender for 5°C storage was given in Section 16-3 (see Table

16-5). Several pilot experiments indicated that semen could also be handled under the moderate ambient temperatures prevailing during the short breeding season in New Zealand. An extensive test (87) gave the following 49-day NR:

	5°C		*Ambient temperature*	
Age of semen	*No. cows*	*% NR*	*No. cows*	*% NR*
Day collected	268,201	65.3	44,354	66.2
Day later	172,663	66.6	29,772	68.9

Because of the good fertility achieved with semen used at ambient temperature, particularly after one day of storage ($P < 0.05$), the majority of inseminations in New Zealand since 1964 have been made with semen kept at this temperature. The optimum temperature range for Caprogen is considered to be 18 to 24°C.

Egg yolk level in Caprogen has been reduced experimentally (87, 138) to as little as 2 percent by volume (see Table 16-10). Five percent egg yolk is used routinely, except for the initial premix with semen, which contains 20 percent yolk. This is consistent with other work (see Table 16-2) showing that less egg yolk is needed with higher temperatures. Furthermore, there appears to be an interaction between spermatozoa and egg yolk levels (87). With low egg yolk levels sperm numbers per insemination can be minimized. The practice for several years in New Zealand has been to extend semen to 4 or 5 $\times$ 10^6 total sperm per milliliter and to use 0.5 milliliter of extended semen per insemination. Earlier work in New Zealand and elsewhere (139)

Table 16-10. Fertility of spermatozoa with different levels of egg yolk in Caprogen extender at ambient temperature.

Total no. inseminations	*% egg yolk*				*Reference*
	20	*10*	*5*	*2*	
	(% 49-day NR)				
9,778	67.7	65.7	68.4	–	(87)
425,456	62.8	–	66.1[a]	–	(87)
16,790	–	–	64.1	66.1[b]	(87)
12,512	–	–	66.9	68.2[b]	(87)

[a]Treatments differ, $P < 0.01$.

[b]Treatments differ, $P < 0.05$.

established that equal numbers of sperm inseminated in 0.5 and 1.0 milliliter of extended semen resulted in similar fertility.

Caprogen contains catalase to decompose the H_2O_2 that is formed as a product of sperm metabolism. This addition consistently and significantly increased fertility by 1 to 2 percent. Examples of results are shown in Section 16-6.

Finally, Caprogen can be used as a liquid extender for resuspending frozen spermatozoa. To accumulate extra semen before the start of the short breeding season New Zealand workers (140) have frozen and accumulated limited numbers of breeding units. The frozen semen is thawed, rediluted with Caprogen, and used at ambient temperature. Fertility of the rediluted semen in three extensive trials was 61.1, 61.1, and 63.3 percent NR. The corresponding regular ambient temperature control results were 62.0, 63.3, and 63.4 percent NR (140). Fertility of the semen collected from individual bulls and then frozen and rediluted was highly correlated with semen collected and used directly. Thus, with proper selection of bulls from which to freeze additional semen, overall fertility would not be appreciably affected.

16-4.4 Coconut Milk. Coconut milk extender (CME) is also used at ambient temperatures. The milk from coconuts had been used as a nutrient medium for the culture of plant embryos and it was combined with other ingredients in an extender for spermatozoa (141). The coconut milk is boiled for 10 minutes and filtered before use. Its formulation, as given by Norman (142), is:

Coconut milk	15 ml
Sodium citrate dihydrate	2.2 g
Sulfanilamide	0.3 g
Egg yolk	5 ml
Distilled water to	100 ml

Included per 100 milliliters of CME are 135 milligrams dihydrostreptomycin sulfate, 10 milligrams polymyxin B sulfate, 1000 units of nystatin and 15,000 units of sterile catalase. For ambient temperatures 0.5 to 1.0 percent egg yolk was suggested (32), but 5 percent egg yolk (v/v) extends the use range of refrigerated semen.

In early fertility tests in the United States CME was found to give results equivalent to skim milk-glycerol and CUE (32). Liveability tests and limited fertility data suggested that spermatozoa might be used for breeding after several days' storage in CME. Semen used after 1 to 5 days in Uganda, Kenya, El Salvador, Paraguay, India, and West Germany gave conception rates ranging from 55 to 72. Malmberg and Israelsson (143) obtained 67.7 percent 84-day NR from 5,760 services with CME used for 4 days at 15 to 25°C, and 65.5

percent from 5,832 services with frozen-thawed semen. When semen stored in CME was used for several days (about 400 services per day) the respective 84-day NR figures for the seven days were 69, 67, 63, 61, 52, 46, and 39 percent. Fertility dropped markedly after the fourth day. CME can be used with equal success for cattle and buffalo semen (144).

Coconut palm sap was studied (145) as a possible extender that could be prepared in convenient freeze-dried form. It was less effective, however, than the coconut fluids in maintaining sperm motility.

Whether coconut fluids provide any materials unique for sperm preservation is debatable. The analytical grade reagents used in CUE provide a medium as satisfactory as the citrate-coconut milk extender for sperm survival over an 8-day period at 23 to 25°C (146). When CUE with 1 percent egg yolk was used to store 15 samples of bull semen there was no detectable decline in motility for 8 days, compared to a decline from 60 percent motile sperm to 38 percent after 8 days with 20 percent yolk-CUE. This emphasizes again the importance of using a low egg yolk level in ambient temperature extenders. The results from other studies with coconut milk (147) revealed that 5 to 15 percent of skim milk and glucose combined with citrate buffer was equal to CME in maintaining sperm motility at 16 to 27°C.

In summary, extenders that are successful at ambient temperature are generally characterized by a balanced buffer solution, low egg yolk content, catalase, and a combination of antibiotics.

16-5 MICROORGANISMS IN SEMEN AND THEIR CONTROL

Microbial contamination of semen may 1) affect spermatozoa directly, 2) compete for substrate in semen extenders, and 3) infect inseminated females. The result can be lowered conception rates and increased embryonic mortality or abortion. Microorganisms can gain entry into the semen from infected bulls, during semen collection, during semen processing, or during insemination. Thus it is extremely important to use sterile equipment, sterile media, and aseptic procedures at all steps of the program. Obviously, it is important to start with a healthy bull, but this is not always possible, since unknown or undetected diseases can exist in a bull. However, organizations that comply with the American Veterinary Medicine Associations/National Association of Animal Breeder's "Code of Minimum Standards for Health of Bulls and Hygiene of Bull Studs Producing Semen for Artificial Insemination" (148) have effectively controlled known infectious diseases that might be transmissible through semen. Continuous vigilance under proper veterinary supervision is essential. It is regrettable that many custom freezing operations on farms and ranches almost totally ignore the code, thus working to the detriment of AI.

Even under the best conditions some microbial contamination can occur. Chemical treatment of semen to inhibit multiplication of these contaminants

or to kill them without damaging the sperm cells is therefore important. Comprehensive coverage of published reports on the microbiology of bull semen and means for control are available (41, 148–151). The intent of this section is to outline the development and current status of our knowledge in this area. Additional references can be found in the reviews cited.

16-5.1 Microflora of Bull Semen. Since Gunsalus et al. (152) called attention to the numbers of bacteria in semen several reports have appeared (34, 153–157). In general, there are usually fewer than 150,000 organisms per milliliter of semen with a range from no detectable organisms to samples with several millions per milliliter. Cleaning the sheath and underline of the bull (146) and placing a small apron on the chest of the bull and a polyurethane device at the entrance to the artificial vagina (158) effectively reduce contamination at the time of semen collection. Second ejaculates of semen have lower average bacterial counts than first ejaculates.

Bacilli, diphtheroids, micrococci, coliform organisms, streptococci, staphylococci, pseudomonas, actinomyces, protus, yeasts, viral agents, and mycoplasma have all been found in bull semen. Many of the organisms present are not pathogenic and there is therefore no direct correlation between fertility and the total number of organisms present. Most types of organisms, if present in excessive numbers, will reduce sperm survival (153, 156), but this is not always true.

One organism that appears to be associated with low fertility in bulls is *Pseudomonas aeruginosa* (152–154, 157). This organism can be transmitted through frozen semen (159). *Pseudomonas aeruginosa* and *Clostridium pyogenes* occasionally infect the seminal vesicles and then are contributed to semen in the vesicular secretion (150). Other organisms, which are contagious and can infect cows through contaminated semen, include *Brucella abortus, Vibrio fetus, Trichomonas fetus, Leptospira pomona, Mycobacterium tuberculosis, M. paratuberculosis* (agent of Johne's disease), *Mycoplasma bovine genitalium,* and viral agents that cause infectious bovine rhinotracheitis (IBR) and bovine viral diarrhea (BVD). International shipments of semen must come from areas free from foot and mouth disease and blue tongue, since the viruses responsible for these diseases are transmissible if present in semen.

Efforts are being made to completely eliminate such organisms from bull studs. Through strict programs of testing, selection, isolation, and treatment the major AI organizations have waged a successful battle against most of the organisms listed. Less is known about methods for preventing infection or curing animals with mycoplasma or IBR.

Simultaneously, continuous research is necessary on chemotherapeutic methods of controlling contaminants in semen. Not only can unknown organisms be present, but known organisms, previously sensitive to antibiotics, can

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develop resistance (160). Refrigeration greatly suppresses the multiplication of most such organisms, but does not necessarily stop it (161, 162). Most can survive freezing.

16-5.2 Antibiotics and Other Antimicrobial Agents. As pointed out in the previous section, even when rigid hygienic measures are imposed variable numbers of organisms are present in fresh bull semen. In the early commercial development of artificial insemination semen was stored at 5°C or higher. Egg yolk provided a good nutrient medium for bacterial growth. Effective agents were needed that were easy to use and would control bacterial growth, be nontoxic to spermatozoa, and increase fertility.

Sulfanilamide. Salisbury and coworkers had found that sulfanilamide depressed the rate of sperm metabolism, increased sperm liveability, and partially inhibited bacterial growth. Fertility tests (62) showed that 0.3 percent (w/v) sulfanilamide in a yolk-citrate extender increased the nonreturn rate about five percentage units, a value that was soon confirmed (60). Not all reports were in agreement, particularly with antibiotics in the extender (57). Subsequent studies indicated that if the extender was mixed with semen before rather than after cooling to 5°C, the beneficial effect of sulfanilamide disappeared. This beneficial effect had been interpreted earlier (62) to be primarily a metabolic one, and the fact that egg yolk during cooling also was effective supported the idea that sulfanilamide enhancement of fertility was not due to bacterial inhibition. Sulfanilamide damages spermatozoa during the freezing process (163), and should be excluded from extenders used for semen that is to be frozen. Other sulfa drugs have been tested for possible superiority over sulfanilamide (162), but none has been found that warrants adoption.

Penicillin and Streptomycin. These two antibiotics were among the first to be researched intensively and are still in use. They were found to be relatively harmless to sperm cells and, particularly in combination, inhibited a broad spectrum of microorganisms (57, 123). Almquist (164) first reported that 500 to 1,000 units of penicillin per milliliter of extended semen increased the fertility of low-fertility bulls. Foote and Bratton (165) obtained higher nonreturn rates when 1,000 units of penicillin per milliliter, or 1,000 micrograms of streptomycin per milliliter, or both, were added to extended semen. Sulfanilamide had no effect. These findings were substantiated by other reports (166, 167). Later most of the beneficial effect of the two antibiotics was reported to be due to streptomycin in either egg yolk or milk extender (168, 169). All American reports showed that the nonreturn rate of low-fertility bulls tended to be raised more by addition of these antibiotics to semen extenders than did that of high-fertility bulls, and that delayed returns were decreased.

An extensive trial by Campbell and Edwards (67) also showed that fertility increased when sulfanilamide, penicillin, and streptomycin were added to yolk-citrate singly or in combination. Streptomycin depressed fertility in yolk-phosphate extender, probably because of a precipitate that was formed as a result of the reaction of the streptomycin calcium chloride complex with phosphate buffer. Easterbrooks et al. (170) observed that this problem can be circumvented by using dihydrostreptomycin. Extensive fertility testing indicates that the different forms of streptomycin give equal fertility.

Penicillin usually is added at levels of 500 to 1,000 units per milliliter and streptomycin or dihydrostreptomycin at 500 to 1,000 micrograms per milliliter of extender. Levels much greater than these have been found by some to reduce sperm survival (57), but levels as high as 4,000 units of penicillin and 8,000 micrograms per milliliter of streptomycin or the dihydro form were reported to be harmless (171). Conception rates were improved with 500 to 5,000 micrograms per milliliter of dihydrostreptomycin added to a yolk-citrate-sulfanilamide extender (172).

Both penicillin and streptomycin have been widely used since about 1950. Their popularity stems from the overwhelming evidence of their beneficial effects on fertility. Even the low percentage of pregnant animals that show estrus and are again inseminated are usually protected from possible abortion. Antibiotics have not shown beneficial effects in all tests, however. The semen in these tests may have been free from pathogens, or the method used to expose the semen to antibiotics may have been ineffective. The fertility response to antibiotic treatment today would probably be less than that originally observed now that many of the organisms, such as *Vibrio fetus* (which streptomycin particularly helped to control), have been eliminated from bull studs. In the Netherlands rigid culling of bulls based on bacteriological examination has long been the rule, and antibiotic treatment of semen has had negligible effects on fertility. On the other hand, Melrose et al. (114) in England reported a 3.4 percent increase ($P < 0.001$) in fertility when semen from *V. fetus*-free bulls was treated with 1,000 micrograms of streptomycin per milliliter.

Other Antibiotics. Many other antibiotics have been screened for effects on spermatozoa (see Table 16-11). Oxytetracycline (20 μg/ml), chlortetracycline (50 μg/ml), and chloramphenicol (500 μg/ml) in individual tests were ineffective in raising fertility (178). Several antibiotics such as the tetracycline series and particularly the fungicidal agents nystatin and amphotericin B are quite spermicidal. Others, such as epicillin, are relatively innocuous to sperm cells but also are ineffective against bacterial contamination. Nevertheless, an array of antibiotics exist that are effective against a variety of organisms. Since they can ordinarily be combined safely at levels that are harmless to spermatozoa when they are used singly, judiciously chosen combinations could inhibit a broad spectrum of semen contaminants. Lincomycin and spectinomycin have

Table 16-11. Nonsp

Antibiotic	*M lev*
Tetracycline (Panamycin)	
Oxytetracycline (Terramycin)	<
Chlortetracycline (Aureomycin)	
	<
Chloramphenicol (Chloromycetin)	
Neomycin	
Polymyxin B	
Erythromycin	
Kanamycin	
Tylosin	
Thiostrepton	
Nystatin	
Amphotericin B	
Colymycin	
Lincomycin	
Spectinomycin	
Epicillin	
Ampicillin	

[a]Tests that were conduc
toxicity may be greatly
[b]YC = yolk-citrate; SN
[c]Foote et al. Unpublish

Table 16-11. Nonspermicidal levels of antibiotics in bull semen extenders.[a]

Antibiotic	*Maximum safe level*	*Kind of extender and temperature*[b]	*Reference*
Tetracycline (Panamycin)	75 μg/ml	Krebs-Ringer, 3 hrs. at 37°C	(36)
Oxytetracycline	200 μg/ml	YC, 8 days at 5°C	(173)
(Terramycin)	<125 μg/ml	SM, 4 days at 5°C	(174)
Chlortetracycline	100 μg/ml	YC, 4 days at 5°C	(154)
(Aureomycin)	62.5 μg/ml	YC, 15 days at 5°C	(171)
	<50 μg/ml	YC, 14 days at 4.5°C	(175)
	<250 μg/ml	SM, 4 days at 5°C	(174)
Chloramphenicol	500 μg/ml	YC, 15 days at 5°C	(171)
(Chloromycetin)	1000 μg/ml	YC, SM, 12 days at 5°C	(c)
	1000 μg/ml	YCG, YTG, months at −196°C	(c)
Neomycin	500 μg/ml	SM, 4 days at 5°C	(174)
	1000 μg/ml	SM, 20 days at 5°C	(176)
Polymyxin B	2000 μg/ml	YC, 14 days at 5°C	(154)
	1000 μg/ml	SM, 4 days at 5°C	(174)
	1000 μg/ml	YCG, YTG, months at −196°C	(c)
	1000 μg/ml	YC, 30 min. at 30°C	(177)
Erythromycin	1000 μg/ml	YC, SM, 12 days at 5°C	(c)
	1000 μg/ml	YCG, YTG, months at −196°C	(c)
Kanamycin	1000 μg/ml	YC, SM, 12 days at 5°C	(c)
	1000 μg/ml	YCG, YTG, months at −196°C	(c)
Tylosin	1000 μg/ml	YC, SM, 12 days at 5°C	(c)
	250 μg/ml	YCG, YTG, months at −196°C	(c)
Thiostrepton	25 μg/ml	YC, SM, 12 days at 5°C	(c)
Nystatin	5 μg/ml	YC, SM, 12 days at 5°C	(c)
Amphotericin B	<1 μg/ml	YC, SM, 12 days at 5°C	(c)
Colymycin	<250 μg/ml	YCG, YTG, months at −196°C	(c)
Lincomycin	1000 μg/ml	YCG, YTG, months at −196°C	(c)
Spectinomycin	1000 μg/ml	YCG, YTG, months at −196°C	(c)
Epicillin	1200 μg/ml	SM, 20 days at 5°C	(176)
Ampicillin	1000 μg/ml	YCG, YTG, months at −196°C	(c)

[a]Tests that were conducted without egg yolk or milk in the medium generally are excluded because toxicity may be greatly different in the absence of these ingredients.

[b]YC = yolk-citrate; SM = skim milk; YCG = yolk-citrate-glycerol; YTG = yolk-tris-glycerol.

[c]Foote et al. Unpublished data. Cornell University. 1963–1973.

been combined to provide such control. This combination, along with clindamycin, is especially effective against mycoplasma (179).

Few fertility tests have been reported in recent years. The addition of polymyxin B has been reported to increase the fertility of frozen semen (180, 181); this is similar to the original report for liquid semen (165). Subsequent studies with both liquid and frozen semen have failed to demonstrate an improvement when added to extender containing penicillin and dihydrostreptomycin (see Table 16-11).

Other Chemotherapeutic Agents. Pyridium, phenoxethol (161), nitrofurans (36, 161), sodium azide (a respiratory poison) (182), and other agents (183) have been added to semen. These have either been relatively toxic to sperm cells at levels that are bacteriostatic, or they have been difficult to use routinely because of low solubility or instability.

16-5.3 Control of Specific Pathogens. Much attention has been given to the control of specific pathogens in semen (57, 148, 149, 182). Although unrecognized at the time, one of the organisms that could be spread widely by semen from infected bulls during the early expansion of artificial insemination was *Vibrio fetus.* Numerous studies (184–187) support the conclusion that dilution and streptomycin treatment of semen prevents vibriosis from being transmitted via AI by *V. fetus* carrier bulls. But Morgan et al. (119) showed that streptomycin treatment of semen extended with egg yolk was ineffective in controlling the spread of infection by AI, whereas in milk it was effective. Albertsen (182) showed that the marked effectiveness of streptomycin against *V. fetus, B. abortus,* and *Corynebacterium pyogenes* was greatly diminished by egg yolk. He recommended incubating the semen with antibiotic for 15 minutes at 37°C or for 60 minutes at 30°C before adding egg yolk. A modification of this incubation procedure (177, 180) with the addition of 1,000 units of polymyxin B plus 2000 micrograms of dihydrostreptomycin per milliliter of raw semen is reported to provide a more effective control of *V. fetus.* It is possible that the antibiotic neomycin would be a better choice to combine with penicillin and streptomycin because several strains of *V. fetus* are sensitive to this antibiotic (36, 174), whereas polymyxin B was less effective (174).

Similar examples could be given for other pathogens but the problems have been discussed thoroughly (148, 149). The fact is that many organisms can survive in frozen semen. Clearly the kind and quality of antibiotic, the time and temperature of exposure of organisms to the antibiotics, and the composition of the extender should be considered in formulating a satisfactory procedure for controlling microorganisms in semen.

The ultimate goal is to eliminate all harmful organisms from the bull stud and, through sanitary procedures, to minimize semen contamination. Even under the best conditions, however, an effective mixture of microbial inhibitors in the semen appears to be good insurance.

16-6 ACTIVATORS, INHIBITORS, AND OTHER ADDITIVES

Early work on many types of additives has been reviewed (57), showing that activation by adjustment of pH has not given consistent results and should not be necessary in a properly buffered extender. The addition of sodium bicarbonate to Caprogen in a breeding experiment with 9,741 cows (87) resulted in 68 percent 49-day NR compared with 67.1 percent for the controls ($P > 0.05$). An analog of thiamine—thiamine propyl disulfide—is reported to penetrate the cell and 10 to 60 ppm added to yolk-citrate at 4°C markedly increased spermatozoa viability (188). Adenosine increased the motility of sperm stored at 4°C in 14G (Table 16-3) extender (189). At 37°C 6 millimoles caffeine stimulated and maintained motility of bull sperm cells without loss for four hours (190). Ascorbic acid is also reported to improve fertility periodically (191). "Implementors," such as posterior pituitary extract (mostly oxytocin) and a parasympathetic nerve stimulant (carbamylcholine), have been tested with the hope of aiding sperm transport. Limited experiments in Russia (91) tend to support their value but the results are often not repeatable (192).

The tranquilizer chlorpromazine, at 200 μg/ml, could replace sulfanilamide and improve sperm motility in egg yolk extender (CU-16), but it is toxic to spermatozoa in skim milk. In extensive field trials fertility was not affected when chlorpromazine was added to egg yolk extender (75). Promazine depressed sperm motility at levels as low as 20 μm/ml. Other tranquilizers have been studied (193) and incorporated into the Japanese commercial liquid semen extender, Neoseminan (95). Carbon dioxide and reduced pH also may inhibit and preserve spermatozoa (see Sections 16-2 and 16-4). At very high sperm dilutions cyanide, a highly toxic chemical that inhibits oxidation, improved sperm survival at 35°C in a fructose-containing medium (43).

Protective agents added include the chelators EDTA (ethylenediamine tetracetic acid) and glycine (89, 194, 195). EDTA also protects against the amino acid oxidase released by dead sperm (196), but when it is added to Caprogen (87) fertility is significantly lower (4.8 percent) than with catalase (7,419 inseminations). Catalase is included routinely in ambient temperature extenders to hasten the decomposition of H_2O_2. In one report catalase alone increased sperm survival but failed to increase fertility except when combined with 8 μg/ml of α-amylase (197). According to several reports from New Zealand, however, catalase increased the nonreturn rate 1 to 2 percentage units as indicated by the following 49-day NR data based upon 669,639 inseminations:

Age of semen	*Caprogen*	*Caprogen + catalase*
Day collected	63.4%	64.6%
Day later	66.0%	68.1%

In another trial (196) catalase increased the nonreturn rate 3.1 percent on 8,154 services. Total sperm numbers per insemination dose in Caprogen plus catalase with 5 percent egg yolk could be reduced, without reducing fertility, from 2.5 to 1.5 $\times$ 10^6.

Catalase improves sperm survival at 5°C as well. The effect is most pronounced at the higher extensions of semen (45, 198, 199). Catalase is superior to peroxidase in protecting bull spermatozoa (199). With semen used for insemination at 5°C catalase (1 to 20 μg/ml) added to CUE failed to improve fertility (40, 85) although there was a suggestion of a beneficial effect on fertility after 4 days' storage (45). Catalase improved fertility in heated homogenized whole milk as indicated by the following data (80).

	No catalase		*1 μg catalase/ml*	
	CUE	*Milk*	*CUE*	*Milk*
No. inseminations	6,064	5,944	6,243	5,586
% 60–90-day NR	72.9	68.0	73.2	71.6

Hyaluronidase was thought to play a role in the sperm's ability to penetrate the egg, but hyaluronidase added to semen failed to give a consistent response (57). More recently α- and β-amylase and β-glucoronidase have been shown to improve sperm motility (200, 201). Fertility tests with frozen semen are described in Chapter 17. In New Zealand tests (13,082 inseminations) with semen handled at ambient temperatures α-amylase alone added to Caprogen gave 63.4 percent versus 64.6 percent 49-day NR for control semen (183). The addition of nicotinamide, which as a competitor of nucleosidase in plasma reduces nicotinamide adenine dinucleotide (NAD), significantly depressed fertility 1.9 percent (87).

Hormones have been added to semen in an attempt to influence possible sperm transport in the female, sperm capacitation in the cow, and ovulation time. When 200 IU of human chorionic gonadotrophin (HCG) were added to semen to influence ovulation time fertility results were poorer (202).

	No. cows	*% 60–90-day NR*
Milk	2,077	64.6
Milk + HCG	1,999	62.8

The administration of female sex hormones estrogen and progesterone to extended semen has also been ineffective in altering fertility (57, 203). In conclusion we can say that catalase is the only additive that has been tested thoroughly and used frequently, particularly when semen is stored at ambient temperatures.

16-7 EXTENSION PROCEDURES AND RATES

Semen should be processed in a manner that will insure maximum fertility. The final extension rate of semen should be planned so that the number of sperm present in a package convenient for handling and insemination will be sufficient but will not waste sperm cells unnecessarily.

16-7.1 Initial Semen Extension. It is important to mix bull semen with a preserving medium relatively soon after semen collection in order to provide spermatozoa with necessary ingredients and to protect them in various ways (Section 16-2). Semen and extender should be combined at the same temperature, usually about 30°C. The possible importance of a short incubation period at this temperature with antibiotics added to insure adequate bacterial control is discussed in Chapter 17. For semen used at ambient temperature the final extension can be made at one time.

When semen is to be cooled to approximately 5°C it is convenient to cool the extended semen in relatively small volumes and then add more cold extender to achieve the final extension rate. This can be accomplished by mixing the neat semen with 2 to 4 times its volume of extender before cooling to 5°C and then adding cold extender. Semen cooled without the benefit of extender was 6.5 percent lower in fertility (55) than semen mixed before cooling, clearly indicating the need for protection against the cold. When sperm cells become cold shocked they exhibit a decrease in motility, reduction in metabolism, loss of key components, and morphological damage.

16-7.2 Cooling Rates. Cooling rate undoubtedly is important (see Chapter 17), but the optimum rate for most extenders has not been tested with regard to fertility. Rapid cooling is generally undesirable. McFee and Swanson (204) found that sperm motility was considerably improved during a 10-day storage period at 5°C when sperm in a yolk-citrate-fructose extender were cooled from 32 to 5°C in 140 minutes compared to 25 minutes. An extensive fertility test with homogenized, sterilized 9 percent cream (205) gave the following results:

Cooling time from 30° to 4°C	*No. cows*	*% 60–90-day NR*
120 minutes	9,163	64.8
40 minutes	8,659	63.0

The slower cooling was definitely preferable ($P < 0.02$).

16-7.3 Storage Temperature. Unfrozen semen (except for that which is stored at ambient temperature) is almost always stored at 4 to 5°C. This temperature can be maintained fairly easily with ice and has been satisfactory for

semen used to impregnate millions of cows. It may not be the optimum holding temperature, however. Swanson et al. (206) found significantly better motility and fertility in a yolk-citrate-bicarbonate-fructose extender used to store semen at 10°C rather than at 4 to 5°C. When 1,338 cows were inseminated with semen stored for 3 to 4 days at 4 to 5°C and at 10°C the 60–90-day NR figures were 67.9 and 73.5 percent respectively. Storage at 10°C has not been used commercially, however, because it is difficult to maintain this temperature under practical conditions.

16-7.4 Final Extension Rate and Sperm Numbers. Semen for use at 5°C is usually extended to give the desired number of sperm cells for insemination in one milliliter. Although there is little difference in fertility when 0.5 to 2.0 milliliters of extended semen is inseminated (139) the volume used most often is 1.0 milliliter. Figure 16-1 shows a summary of work by Salisbury and co-workers and Willett and others prior to 1960. In the pioneer work by Salisbury (207) fertility sometimes appeared to increase as semen was extended at higher

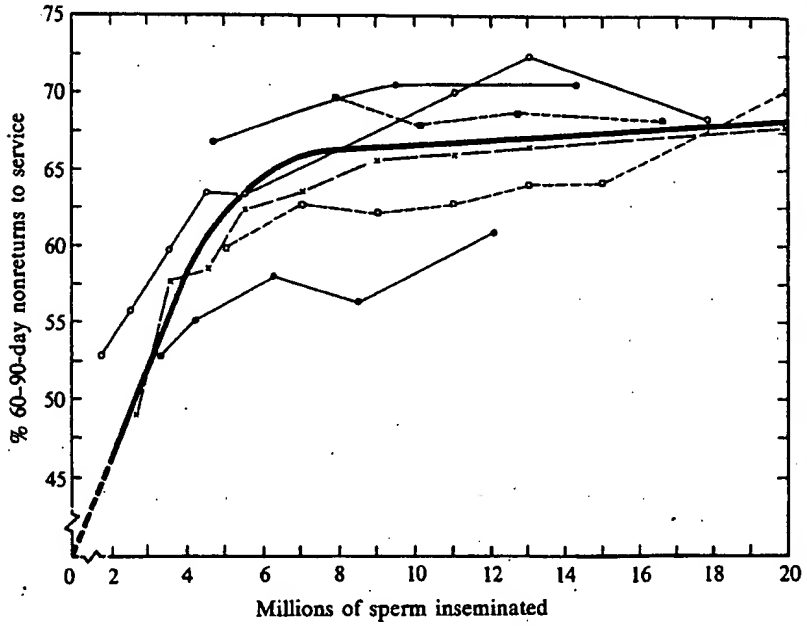

Figure 16-1. A summary of several of the investigations made to determine the minimum number of spermatozoa for optimum fertility in cattle. Most of the experiments were conducted with yolk-citrate as the diluent, with and without sulfanilamide and antibiotics. [From Salisbury and VanDemark. *Physiology of Reproduction and Artificial Insemination of Cattle.* 1st ed. W. H. Freeman and Company, San Francisco. 1961.]

rates up to 1:100. Dilution may have helped to control bacterial contaminants. As Figure 16-1 shows, there is a curvilinear relationship between number of sperm inseminated and fertility. The data are based upon extending the spermatozoa at different rates regardless of initial sperm concentration. This gives a range of sperm concentrations per milliliter used for insemination. Willett and Larson (167) calculated that there was a 0.6 percent decline in nonreturn rate for each decrease of one million sperm inseminated as the numbers diminished from 12 to 4 million. Below 4 million the nonreturn rate declined 3.9 percent for each reduction of one million spermatozoa. In general, an extension rate of more than 100× was possible before too few sperm were present for maximum conception rates. Bratton et al. (208) took initial sperm cell concentration and motility into account and extended samples to 5, 10, and 15 million motile cells per milliliter. This resulted in 60–90-day NR figures of 66.7, 70.9, and 70.5 percent respectively. Therefore 10×10^6 motile sperm were recommended for insemination.

Since 1960 the number of sperm per insemination necessary to maintain optimum fertility with liquid semen has decreased further. This has been made possible by the development of more balanced extending media. Results of several trials in this development are shown in Table 16-12. The trials at 5°C indicate that 4 million motile sperm per milliliter gave initial high fertility and that when the number per insemination was decreased to 2×10^6 fertility changed little (109). A report by the British Milk Marketing Board showed that extremely high dilutions of semen from two bulls up to 1:3,000—giving 500,000 live sperm per milliliter—resulted in 32 and 46 percent conception. When sufficient sperm are inseminated increasing the number further does not improve the fertility of poor quality semen (210) or low-fertility bulls (15), nor does it compensate for the reduced fertility caused by poor technicians (87). (The latter effect was studied at ambient temperature.)

In ambient temperature studies (see Table 16-11) 12.5 to 25×10^6 sperm per insemination originally were employed. This number was reduced drastically and fertility remained satisfactory. Reducing the proportion of egg yolk in extender from 20 percent to 5 percent facilitates extending to very low numbers of sperm. Shannon (138) observed that fertility declined 10.9 percentage units when the sperm dose in 20 percent yolk-Caprogen was reduced from 2.5 to 0.5×10^6 sperm per insemination. The corresponding decline with 5 percent egg yolk was only 3.1 percentage units. This decline is probably due to a reduction in sperm numbers below the critical level, although high dilutions per se could be detrimental. Since the volume inseminated in New Zealand is 0.5 milliliter the actual semen dilution is one-half of what would be required if one milliliter were used. Nitrogen in the Caprogen extender also reduced the dilution effect (46).

The enormous impact that a single sire could have on the gene pool in a liquid semen program, if enough cows were available to inseminate with

Table 16-12. Fertility of unfrozen semen with different numbers of spermatozoa per insemination.

Temperature and treatment	*No. 1st services*	*% 60–90-day NR*	*Reference*
5°C, motile sperm			
CUE, 5×10^6 sperm	24,737	75.2	(5)
CUE, 10×10^6 sperm	18,933	76.1	
CUE, 4×10^6 sperm	11,065	72.7	(15)
CUE, 8×10^6 sperm	9,739	73.4	
Tris, 4×10^6 sperm	11,445	72.7	
Tris, 8×10^6 sperm	10,071	74.1	
Ambient; total sperm[a]			
Caprogen, 5×10^6	15,997	68.0	(196)
Caprogen, 3.75×10^6	8,574	69.3	
Caprogen, 2.5×10^6	8,922	67.0	
Caprogen, 3.75	14,110	63.6	(196)
Caprogen, 2.5	6,130	63.3	
Caprogen, 1.875	6,124	61.2[b]	
Caprogen, 2.5	12,676	66.8	(87)
Caprogen, 0.5	4,114	59.5[c]	

[a]Sperm concentration/ml is twice that given; 0.5 ml is used for insemination.

[b]$P < 0.05$.

[c]$P < 0.01$ within experiments.

0.5×10^6 total sperm, is obvious. About 30 billion or more sperm per week can be collected from most mature healthy Holstein sires. This number of spermatozoa is sufficient to inseminate 6,000 cows per week. In New Zealand 2.0 to 2.5×10^6 total sperm are routinely used per insemination, except when frozen semen has been banked for later thawing and used as needed at ambient temperatures. In this case, the semen is thawed and reconstituted in Caprogen with 20 million sperm per insemination dose.

16-7.5 Packaging Unfrozen Semen. All semen should be packaged in opaque containers for transport and storage in order to prevent exposure to light (33). In moderate climates where ambient temperatures are used the semen is often wrapped in aluminum foil and placed in light-weight styrofoam containers. The containers should be closed and kept out of direct sunlight.

16-8

For semen stored at 3 to 5°C a plastic bottle of frozen water included in a styrofoam or other insulated container will keep the cells at this temperature as long as there is ice in the water. The bottle of frozen water should be removed from the freezer, where it has been held several degrees below freezing, to allow it to warm up from freezer temperatures to 0°C before placing the ice and semen together in the container. It is safe to combine them as soon as the ice in the bottle starts to melt. If the ice bottle is not allowed to defrost it could be cold enough to freeze and damage the spermatozoa.

A similar danger is encountered in winter when the inseminating tubes may be chilled by exposure to below-freezing temperatures. Cold shock results when the diluted semen held at 5°C is moved from its container into inseminating equipment that has been chilled by exposure to freezing weather conditions.

If the temperature of the diluted semen rises during shipment, more ice or more insulation or both are needed. It is not always possible to add more ice to the package, in which case containers with better insulation are required, especially in hot weather.

16-7.6 Warming Rate. After extended semen has been stored at approximately 5°C the rate of warming the cells to body temperature is not critical. The temperature can be raised very rapidly from 5°C to body temperature without harming the cells.

16-8 PROCESSING SEMEN FOR SEX CONTROL

Innumerable reports have appeared concerning alteration of the sex ratio (211, 212). Limited control appears to be possible, but no experimental data have been found to support claims by commercial concerns that they are able essentially to guarantee the sex of the calf produced.

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17

Principles and Techniques of Freezing Spermatozoa

17-1 INTRODUCTION

All life proceeds in a continuous stream, the individuals in it constituting a part of the linkage that has joined past and present life forms and will ultimately join present life forms with those of the future. The life span of each link in the chain is limited by its genetic makeup and its environment, some part of which is controlled by the metabolic rates of the individual links. Among the processes that control the metabolic rate at which spermatozoa exhaust themselves is the temperature at which their life-sustaining chemical processes proceed. These chemical processes generally take place within a measurable temperature range and vary within that range in direct response to the temperature.

Water is the primary constituent of biological fluids responsible for the internal transport of essential chemicals. Pure water freezes and forms crystals at 0°C, whereas water containing ions and other substances in solution freezes at lower temperatures depending on the concentration of these substances. As water in a solution is frozen, pure water crystals form leaving behind greater liquid concentrations of those substances in solution. This increases the osmotic pressure of the remaining solute, which can damage the cells.

British workers discovered that glycerol protected spermatozoa against some of these hazards when it was added to the extender solution in which the spermatozoa were suspended. Since then, freezing has been the preferred method of preserving spermatozoa.

Spermatozoa can now be stored in liquid nitrogen at temperatures as low as −196°C and survive with relatively high fertility after thawing. However, many of the contained spermatozoa are killed or rendered immotile by freezing and thawing. Hence, for optimum fertility larger numbers of spermatozoa are used for frozen than for unfrozen semen.

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Egg yolk and/or milk are the basic ingredients of most extenders for semen freezing. Other protective compounds similar to glycerol have been tested and found unsatisfactory in one way or another. The fertility of frozen semen depends greatly on the care exercised in the initial extension of the semen before freezing, the method of adding the glycerol, the time permitted for equilibration of the spermatozoa with the glycerol, the rate of freezing, and the stability of the temperature during storage. After storage fertility depends on the care and method of warming the extended semen before insemination. When proper procedures are used fertility rates equal to those of any other process can be maintained for extended periods.

Although many of the procedures for freezing spermatozoa are relatively simple, they must be performed correctly if the highest potential fertility for insemination is to be maintained. This chapter will acquaint the reader with the present state of the art of preserving bovine semen by freezing and will relate that to the latest research dealing with the process.

17-2 THEORETICAL ASPECTS OF FREEZING AND THAWING

17-2.1 Physical and Chemical Consequences. At least 50 percent of the sperm from an unselected population of bulls are killed or rendered immotile during freezing and thawing. The damage is caused 1) by internal ice crystal formation that affects the structure of the spermatozoa, 2) by the solute concentration that results as pure water is withdrawn from suspension media both inside and outside the cells, and 3) by the interactions of these two physical factors. This theory of cellular damage is widely accepted among cryobiologists as a working hypothesis in research in this field.

The major physical and chemical consequences of freezing are the removal of pure water from solution to form ice and the resultant increased concentration of solutes in the residual liquid. These events and their effects on the cells are influenced by the level and types of cryoprotective agents, the osmolality and pH of the extender, and the freezing rate (1, 2, 3, 4). Spermatozoa to be stored in glass ampules have generally been frozen at relatively slow rates. The range of freezing rates employed has been about 0.8 to 3°C per minute from +5 to −15°C, and 3 to 5°C per minute from −15 to −79°C. These rates result in the formation of a relatively small number of large extracellular ice crystals.

The freezing point of a solution is based on the concentration of particles it contains as solutes. A given molecular weight of an electrolyte that dissociates into ions on entering into solution will lower the freezing point more than the same proportionate weight of a molecule that does not dissociate. As a solution cools to, through, and past the point at which ice forms, a continuing series of events takes place. The rate of cooling depends on the difference in temperature between the solution and its coolant and their relative

mass. Depending on the concentration of solutes and the rate of cooling, the temperature of the solution falls below the true freezing point of water until an ice crystal forms around a "seed." At that point the heat of fusion required to form ice must be removed from the solution, so its temperature shifts upward as the process takes place. As each molecule of pure water freezes out of the solution, the concentration of particles in that which remains increases, resulting in a continuous drop in the freezing point of that solution. These interrelated changes continue until the entire solvent and its contained solutes and other contents have completed the process through the eutectic point. Due to the longer exposure of the cells, the damaging effects of high solute concentrations should be most critical with slow rates of freezing. Similarly, because cells may be exposed to damaging solute concentrations during rewarming, fast thawing should be beneficial.

Although the formation of extracellular ice crystals was once considered an important disruptive force it does not seem to be a primary cause of sperm-cell damage during freezing (6, 7). Extracellular ice is not, in fact, particularly harmful (6–9). With few exceptions (10, 11), however, intracellular ice is lethal (1, 3, 5, 6). In addition, recrystallization—the growth of large crystals at the expense of smaller ones—can occur at temperatures above −130°C (2). Ice crystals can also form and cause cell damage as stored samples are warmed from −196°C.

The increased solute concentrations created as water freezes appear to be a major cause of cell damage. For example, hemolysis of erthyrocytes occurs at electrolyte concentrations above 0.8 M NaCl regardless of temperature (12), and high salt concentrations damage bull spermatozoa (13). During freezing the temperature at which bull sperm were damaged was found to be lower in a hypotonic extender than in an isotonic one (14). Solute concentration, the rapidity of its change, and the damaging effects on spermatozoa depend on temperature (3, 5, 8) and its rate of change. During slow freezing, the cells are exposed for prolonged periods to concentrations of solutes that are increasing relatively slowly and some cellular dehydration may occur. With rapid freezing the length of exposure to increased solute concentrations is reduced and intracellular dehydration may not occur.

The increase in osmotic pressure of the suspension medium that accompanies freezing pulls water from within the cell, causing intracellular dehydration with an attendant increase in intracellular electrolyte concentration. In contrast, rapid freezing, such as that obtained by direct immersion in liquid nitrogen with cooling rates that probably exceed 200°C per minute, results in the formation of a relatively large number of small crystals both within the spermatozoa and in the suspension fluid. Intracellular ice can form during rapid freezing because there is insufficient time for all freezable water to diffuse from the cell (4, 5). Both rapid and slow freezing cause cellular damage, although different mechanisms appear to be responsible.

Changes in pH have been observed during freezing caused by precipitation of salts in saturated solutions (15, 16). This could influence cellular survival, although under normal conditions it seems unlikely that solutions sufficiently saturated to change pH so as to harm the spermatozoa would be produced until after their own metabolism had been greatly slowed by the drop in temperature.

The exact nature of total freeze-thaw damage is not fully understood. It has been suggested that freezing damage is a consequence of the vapor pressure differential between ice and water at temperatures below 0°C. The theory is that the greater vapor pressure of the water would cause its redistribution, leading to injury from dehydration during slow freezing, and to membrane rupture during rapid freezing (22). There is considerable evidence that cellular membranes are involved. Saacke and Almquist (17) observed loss or damage to the cellular membranes of spermatozoa frozen without glycerol, and Rapatz (18) observed damage to the sperm neck, due to intracellular ice, following rapid freezing. Pickett and Komarek (19) found that lipid was lost from spermatozoa during freezing, presumably leaching from cellular membranes. Membrane damage may also account for the increased leakage of sperm enzymes following freezing without glycerol (20). Decreased mitochondrial enzyme activity has been noted following the freezing of mouse liver (21). Such changes are presumably the results of increased solute concentrations that induce protein denaturation.

17-2.2 Glycerol Function. The cryoprotection afforded when glycerol is added to seminal extenders was originally attributed to changes in the size and shape of ice crystals formed (6, 9, 18)—changes that presumably reduced their mechanical destructiveness. A more popular hypothesis states that glycerol acts through a "salt-buffering" mechanism (12). Since glycerol binds water (3, 23, 24) and markedly decreases the freezing point of solutions, less ice forms in its presence at any given temperature. Consequently, the solute concentration in the residual liquid is correspondingly reduced. The damaging influences of concentrated solutes appear to depend on temperature (3, 6, 8); hence glycerol, by reducing the temperature at which such solute concentrations are attained, presumably reduces their damaging effects. In support of this concept, the optimum glycerol level of an extender increases as extender tonicity is increased (25, 26, 27). Glycerol probably dehydrates the cells (34, 35) and it forms complexes with metallic ions (33).

Although cellular damage can be caused by concentrated solutes, other unknown sources of damage presumably exist, and knowledge of the site of glycerol action is essential to a critical evaluation of both cryoprotective mechanisms and the nature of freezing damage.

It has generally been believed that glycerol enters live spermatozoa suspended in it and does so rapidly. Glycerol is oxidized by sperm cells (28, 81) and it appears in autoradiographs to concentrate in the posterior part of the

head (29). The rate of entry is not known, however; nor is it known whether glycerol buildup within the cell is necessary for protection against freezing damage (31, 35). The early view in support of an intracellular glycerol requirement was based on work with red blood cells (12), but recently has been shown not to hold in tests of bovine sperm that compare their freezability after momentary and 30-minute exposure to glycerol before freezing (35). Spermatozoal survival was greatest in one study (34) when they were frozen after only momentary exposure to glycerol rather than after 30 minutes' to 6 hours' exposure. Bull sperm cells have been successfully frozen in the presence of cryopreservatives that do not enter the cell if the freezing rate is very rapid (32). To be most effective, glycerol requires relatively slow freezing rates.

Since spermatozoa act like osmometers, swelling in hypotonic extenders as water enters the cells and shrinking in hypertonic extenders as water is withdrawn from them, it was thought that this technique could be used to determine the rate at which glycerol entered into spermatozoa. The presence of 7 percent glycerol in an isosmotic extender containing a soybean protein decreased spermatozoan volume. In two concentrations of citrate dramatically different in osmolality, in the presence or absence of egg yolk, the packed cell volume was increased by 7 percent glycerol. The change recorded was as great 5 minutes after as it was 30 minutes after the addition of glycerol and was, of course, greater in the hypoosmotic extender (30).

17-2.3 Other Cryoprotective Agents. Glycerol for the freezing of bull semen remains the standard of comparison for any cryoprotective agent. No substitute has yielded results as satisfactory. A number of related compounds, including ethylene glycol and propylene glycol have been investigated (36, 37, 38). Dimethyl sulfoxide (DMSO) penetrates cells rapidly, has a "salt-buffering" effect, and is most effective during slow freezing. A number of disaccharides and trisaccharides have also shown some cryopreservative effects. They do not penetrate the cell and seem most effective when used with relatively fast freezing rates (32). A number of theories have been advanced to explain the cryoprotective effects, most of which have to do with elimination of electrolyte damage (39), cell membrane permeability (40), possible preferential leakage of solutes (41), and buffering by nonelectrolytes (32).

17-3 EXTENDERS FOR FREEZING

17-3.1 Basic Components of Extenders for Freezing. The basic components of extenders for freezing bull spermatozoa are: 1) water (42) which is a solvent for seminal and extender components; 2) dissolved ionic and nonionic substances to maintain osmolality and to buffer the pH of the medium; 3) organic materials with the capacity to prevent cold shock (generally egg yolk or milk); 4) cryoprotective agents such as glycerol or DMSO; 5) simple sugars for an

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6) additives such as
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Buffers and Nonionic Substances. A variety of buffers and nonionic substances have been used, among which are sodium citrate dihydrate (43, 44, 45), Tris (hydroxymethyl) amino methane (27, 46), monosodium glutamate (47), buffers with combinations of salt solutions (48) or carbohydrates (32, 35, 44, 49–52), and various milk preparations (25, 45, 46, 53, 54). Although a number of zwitter ionic buffers have received recent attention (55, 56), yolk-citrate remains the most widely used seminal extender. Reports of superior survival and fertility in other extenders are numerous (47, 51, 52, 54), but possible interactions with other factors in seminal processing make it impossible to generalize these findings; a satisfactory extender for one animal or one set of conditions may not be acceptable for others. Thus each laboratory must determine which extender, under its conditions, will provide maximum reproductive efficiency. It should be emphasized that almost all recommendations regarding minimum sperm numbers, the effects of long term storage, and so on, are based on results with yolk-citrate extenders. Each promising new extender or additive should be carefully evaluated before it is adopted for general use.

Organic Materials. Since Phillips (57) discovered that egg yolk was a valuable constituent in preserving fertility it has been used extensively in seminal extenders. VanDemark et al. (37) investigated the influence of egg yolk levels on the post-thaw motility of spermatozoa in a citrate extender; the results are shown in Figure 17-1. The highest survival was obtained with 24.2 percent egg yolk, although a second experiment showed that 16 percent egg yolk was slightly better than 24 percent. Levels of approximately 20 percent egg yolk have become standard in most extenders.

Post-thaw motility (58, 59), sugar utilization (59), lactic acid production (59), and fertility (58) were greatest when egg yolk was included in both fractions of the seminal extender—that containing no glycerol (which is added for cooling above freezing) and the remainder containing glycerol (which is added before the below-freezing cooling phase is begun).

Extenders have also been developed that consist primarily of heated skim milk and glycerol. With reconstituted skim milk the optimum combination of these components was 10 percent dry milk solids to 9.7 percent glycerol. With fresh skim milk, 10 to 13 percent glycerol was best (25, 54).

The beneficial effects of egg yolk and milk for preventing cold shock prompted research on the nature of the active constituents. It was found that egg yolk does not permanently alter the spermatozoa (60). The optimum levels of its protective constituents—lipoprotein, phospholipid, and lecithin (60–65)—in extenders range from 1 to 5 percent lipoprotein, 0.5 to 1.0 percent

phospholipid, and 0.25 to 2.0 percent lecithin (see Figure 17-2). The milk protein—casein—is the agent in milk responsible for preventing cold shock (62, 65, 66) and its optimum levels have ranged from 1 to 8 percent. The nature of cold shock prevention by these agents is not understood.

Cryoprotective Agents. Glycerol is the most widely used cryopreservative for bull spermatozoa. When frozen by conventional methods in glass ampules, approximately 7 percent glycerol is optimal for citrate-yolk and Tris-yolk extenders; 11 to 13 percent for fresh and reconstituted skim milk. The optimum glycerol level was lower with the pellet method of freezing (35, 51, 67). In fact, yolk-lactose and yolk-raffinose extenders without glycerol resulted in a 50.5 and 59.3 percent 60-to-90-day nonreturn (NR) on 32 and 59 first service inseminations, respectively. Partial cryoprotection (51, 67) was contributed by these sugars and they complemented glycerol when used in combination. When used individually glycerol was superior to dimethyl sulfoxide (DMSO) (68–70). Combinations of these cryopreservatives were not beneficial in homogenized and skim milk extenders. The addition of 1 percent DMSO to a yolk-Tris extender with 6 percent glycerol improved post-thaw motility. In a 7 percent glycerol-Tris-yolk extender, additions of 0.5 to 2.5 percent DMSO did not significantly improve liveability but favored the combined use of these cryopreservatives.

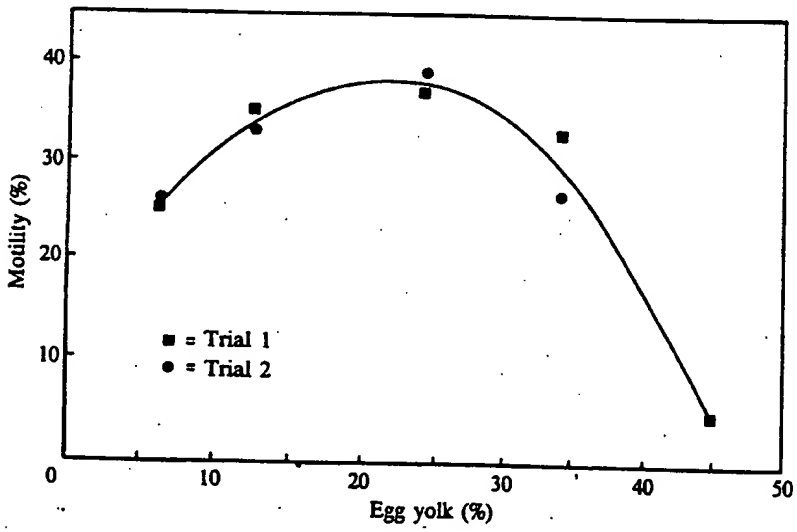

Figure 17-1. Percentage of motile spermatozoa after freezing and thawing of semen in diluents containing various levels of egg yolk. [VanDemark et al. Illinois Agr. Exp. Sta. Bull. 621. 1957.]

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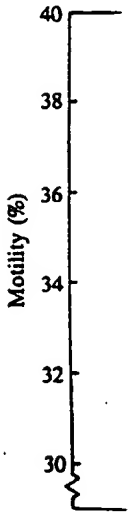

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17-3.2 Sugars. The monosaccharides—glucose and fructose—are sugars from which spermatozoa may derive energy for their life processes; they have been studied extensively as adjuvants to extenders for freezing spermatozoa (25, 46, 54, 58, 64, 83, 84, 85). Some improvement has been observed from their addition, which suggests that the improvement resulted not only from providing immediately available substrate (54) but from providing substitutes for electrolytes in maintaining the osmotic balance of the extender. A level of 4 percent fructose was excessive in one investigation (83). It does not seem to matter whether these sugars are added to either fraction of the extender used for cooling or freezing (48), but they may affect optimum equilibration time (46, 85).

The di- and trisaccharide sugars, however, which spermatozoa are not able to metabolize, provide their cryoprotective benefits primarily as relatively large molecules that contribute to osmotic balance by acting as substitutes for electrolytes. They are not capable of passing across the sperm-cell membranes during freezing or thawing and they probably diminish the effects of solute concentration. They give best results when added to semen in the glycerol-containing fraction before freezing and they are not contained in the extender

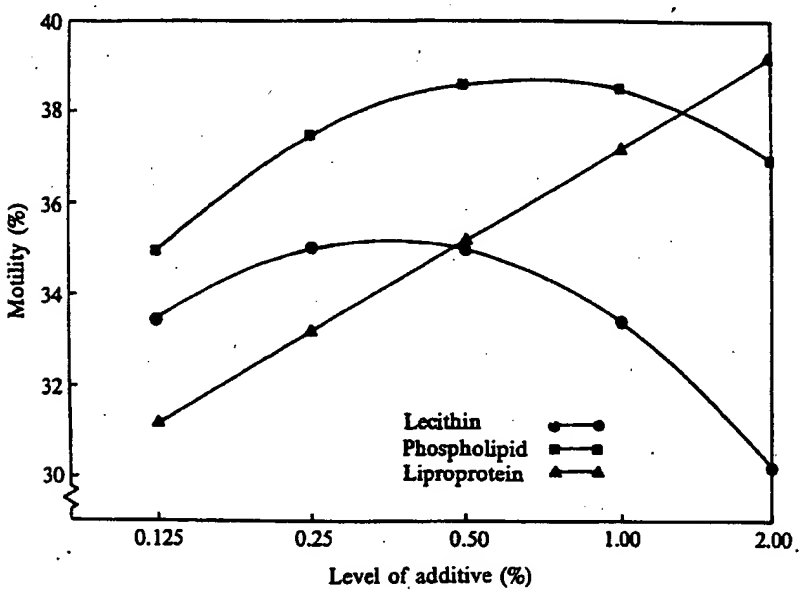

Figure 17-2. The post-thaw motility of spermatozoa extended in Tris-buffered lecithin, phospholipid, and lipoprotein extenders. [From Gebauer et al. *J. Dairy Sci.* 53:817–823. 1970.]

used for prefreeze cooling from body temperature (58). For pellet freezing, in which the rate of freezing is rapid, these sugars replace effectively the buffering salts of extenders (67) and provide cryoprotection for all (35) or part of the replaced glycerol (44) in tests based primarily on post-thaw motility. The fertility of semen frozen with sugar as the cryoprotective agent in yolk was lower than when some glycerol was included (32). The sometimes conflicting results reported in the literature involve differences in equilibration times, rates of freezing, and the kind of seminal containers used in freezing. The protection afforded by the large sugar molecules is different from that provided by glycerol and the two are not interchangeable. The data indicate that at this stage in our understanding glycerol is indispensable and sugars are sometimes complementary and useful.

17-3.3 Osmolality. Osmolality—the number of particles suspended as solutes in a solution—influences not only the osmotic pressure of a solution but the point at which the solvent freezes. Thus the lowered freezing point of a solution reflects its osmolal concentration of particles. An osmolal concentration of a solute in water depresses its freezing point 1.86°C. This is usually expressed as milliosmols; one milliosmol depresses the freezing point 0.00186°C. The freezing point depression of bull semen is approximately −0.55°C, equivalent to a concentration of about 300 milliosmols, which increases as the spermatozoa metabolize nutrients (71, 73). Thus evidence indicates that it is better to err on the side of hypotonic than hypertonic extenders (77). Results of a number of studies show that seminal extenders that are isosmotic with semen preserve the life of spermatozoa best when stored above or below freezing temperatures (14, 27, 71, 74, 75). Figure 17-3 illustrates the results of freezing semen in yolk extender made up of varying levels of sodium citrate. Clearly the effects of osmolality on spermatozoan liveability and ultimate fertility interact with a wide range of other variables, including the hydrogen ion concentration measured by pH (27, 76).

17-3.4 pH. The optimal pH for a seminal extender is influenced by its nature and by the ions it contains. The optimal pH for a phosphate buffer is 7.5 (76), and that for Tris and citrate extender is 6.5 to 7.0 (27) (see Table 17-1). The optimal pH appears to differ, however, at different stages of the semen processing procedure. Steinbach and Foote (27) noted that the least damage before freezing occurred in an extender at pH 6.5, but the smallest loss of spermatozoan motility during freezing was observed in an extender with a pH of 7.5. This was consistent with earlier findings of Pickett et al. (79). With unfrozen spermatozoa a decrease of 0.1 pH unit in a CUE extender increased the NR figures by 2.58 percent over the pH range shown in Figure 17-4. Freezing injury may be reduced in the presence of lower concentrations of hydrogen ions than are optimal for storage in the unfrozen state, and in Tris buffer high

pH was more toxic at low than at high osmolality of the buffer (27, 76). The pH may change during storage but the change bears little relationship to post-thaw motility (76, 80).

17-3.5 Enzymes. Spermatozoan capacitation (see Section 4-6.1), which occurs naturally in the uterus preparatory to fertilization, may occur at least partially *in vitro* by incubation with enzymes hydrolyzing long-chain carbohydrates (86). One of these enzymes is amylase, which is available commercially in either the alpha (α) or beta (β) forms. Most of these discoveries, and tests of the fertility of semen treated with amylase, have been made by Hafs

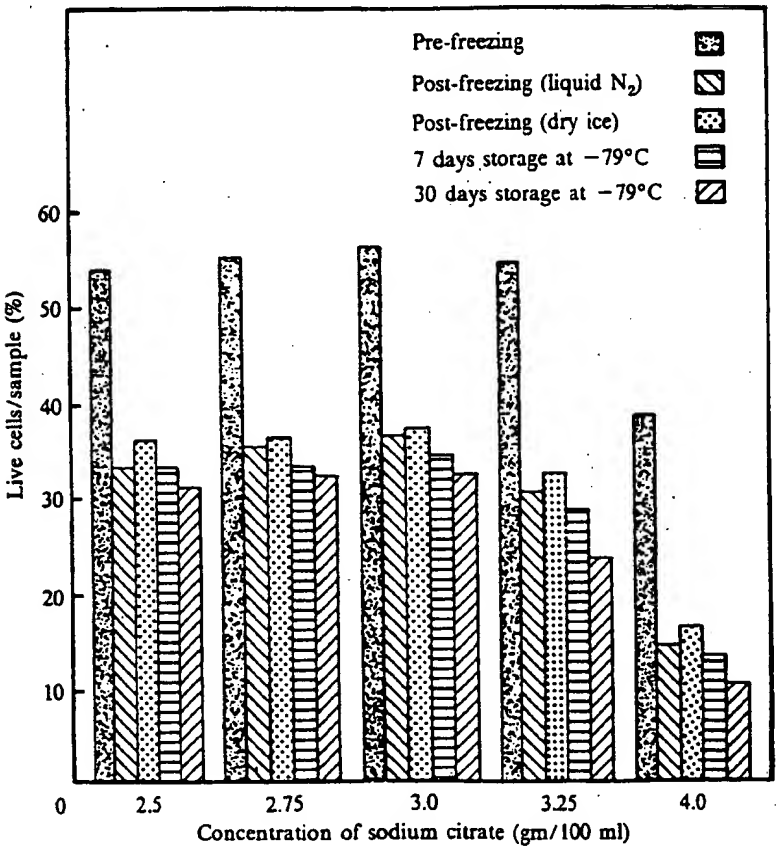

Figure 17-3. The influence of sodium citrate concentration upon the percentage of live spermatozoa at various intervals during processing and storage. [From Rao et al. *Fert. and Ster.* 19:129–136. 1968.]

Table 17-1. The influence of processing techniques and pH on fertility of bovine spermatozoa frozen in Tris and citrate extenders.[a]

pH	Item	Tris[b] I	Tris[b] II	Tris[b] III	Total or mean
6.5	1st services	1,363	1,491	1,083	3,947
	% NR	74	73	74	74
6.75	1st services	1,320	1,499	1,165	3,984
	% NR	73	72	72	72
		Citrate[c] I	*Citrate*[c] II	*Citrate*[c] III	
6.5	1st services	2,615	2,454	2,464	7,533
	% NR	70	72	69	70
7.0	1st services	2,516	2,478	2,438	7,432
	% NR	70	70	69	70

[a] From Foote. *J. Dairy Sci.* 53:1478–1482. 1970(78).

[b] I. All samples glycerolated upon cooling and frozen 6 hours later.
II. Extenders contained glycerol initially.
III. Extenders contained glycerol initially, and samples were frozen 3 hours after cooling.

[c] I. Four-time glycerolization immediately after cooling.
II. One-time glycerolization immediately after cooling.
III. One-time glycerolization 5 hours after cooling (1 hour before freezing).

and his associates at the Michigan Agricultural Experiment Station. Additions of from 1 to 20 micrograms of crude α- or β-amylase generally increased fertility (87, 88, 89), although contrary observations have been reported (228, 230) (see Table 17-2). The optimum levels of amylase additions have not yet been established and may be different for the two forms of the enzyme (90). The improvements recorded have been attributed to increasing availability of metabolic substrates (88, 89). They are most likely due to the shortening of the time necessary for capacitation to occur in the uterus, especially when inseminated in cows bred too late in estrus for normal capacitation to have occurred before ovulation (88, 91).

Beta glucuronidase, another of these enzymes, has resulted in important fertility increases when added to stored semen. The addition of 10, 30, or 150 Fishman Units of β-glucuronidase per milliliter of extender increased fertility

of semen in 1.0-milliliter ampules by 0.3, 1.2, and 5.9 percentage points, respectively (90) (see Table 17-3). The fertility response was almost linear, with 150 units being higher than either 10 or 30 units. O'Hagan et al. (229) compared 0 or 150 units of β-glucuronidase, with and without 10 micrograms of β-amylase per milliliter of semen packaged in 0.5-milliliter straws. The fertility responses with amylase were 1.9 and 1.8 percentage points above the controls at 28 and 112 days respectively, compared to 0.3 and 2.0 for β-glucuronidase. When both enzymes were added, fertility was improved 2.0 and 4.1 percentage points over the controls at 28 and 112 days after insemination, respectively. At 112 days there was a significant increase in fertility from the addition of β-amylase ($P < 0.1$), β-glucuronidase ($P < 0.05$) or both enzymes ($P < 0.001$).

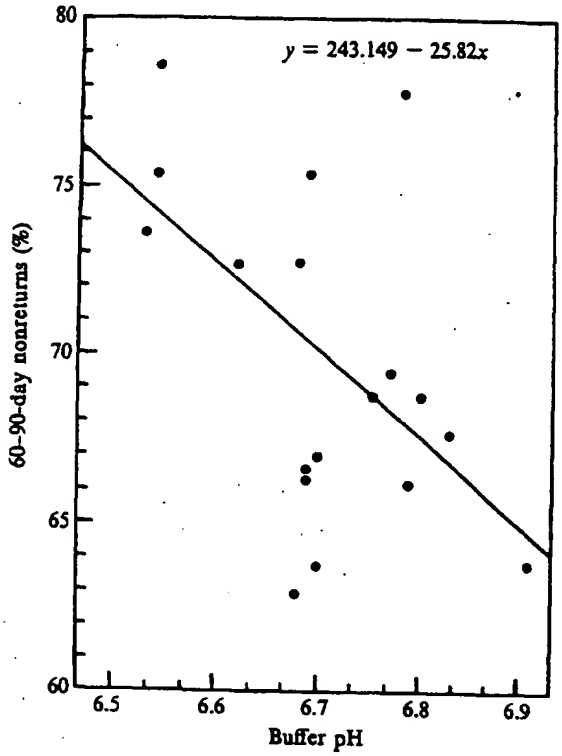

Figure 17-4. The effect of buffer pH on the fertility of liquid semen. [From Pickett et al. Conn. (Storrs) Agr. Exp. Sta. Bull. 373. 1962.]

Although the fertility response appears to be more favorably consistent to the addition of β-amylase than to the addition of β-glucuronidase, it has been postulated (229) that each additive contributes by hydrolyzing complex polysaccharides on the sperm-cell membrane. Contradictory fertility results have been obtained by others, however, (229, 246), so that final conclusions about the use of these enzymes, their amounts, and the best mode of action await further evidence.

Table 17-2. The effect of amylase on percent nonreturn[a] using frozen semen.

Level of amylase	*Type of amylase*		*Ref.*
	Alpha	*Beta*	
(μg/ml)			
0	——68.1(4,294)[b]——		(88)
1	69.0(3,807)	71.1(4,684)	
10	72.1(4,770)	70.7(4,336)	
0	——67.1(1,414)——		(87)
1[c]	68.6(1,932)		
10	69.4(4,623)		
100	65.0(3,619)		
0	——72.0(3,401)——		(90)
10	74.3(1.504)		
20	74.2(2,057)		
0	——72.9(307)——		(228)
10	69.8(318)	70.2(323)	
0	——73.4(6,152)——		(89)
1	75.3(11,492)	75.5(11.329)	
10	75.1(12,084)	75.2(12,237)	
0[d]	——63.1(2,000)[e]——		(229)
10[d]		64.9(2,000)[e]	

[a]60–90-day, except d.

[b]Number of first services in parentheses.

[c]Highly purified, equal to about 10 μg crude amylase.

[d]112-day.

[e]Approximate.

Table 17-3. The effect of enzymes on percent nonreturn using frozen semen.

Level of enzyme				
β-glucuronidase	*Amylase*	*Inseminations*	*% NR*	*Ref.*
(μ/ml)	(μg/ml)			
0	0	3,401	72.0[a]	(90)
10	0	1,961	72.3	
30	0	1,912	73.2	
150	0	1,734	77.9	
0	0	2,000[c]	63.1[b]	(229)
150	0	2,000	65.1	
0	10	2,000	64.9	
150	10	2,000	67.2	
0	10	4,651	77.8[a]	(246)
30	0	4,500	77.2	
30	10	4,337	76.1	

[a]60–90-day.

[b]112-day.

[c]Approximate.

17-4 PREPARATION FOR FREEZING

17-4.1 Initial Extension Ratio. It is common practice to partially extend semen soon after collection to a constant spermatozoal number or to a standard volume for cooling to 5°C. After cooling, sufficient additional extender generally is added to provide twice the final number of sperm desired for insemination. The partially diluted semen is then glycerolated by the addition of an equal volume of extender that contains twice the final desired concentration of glycerol. The authors are aware of only one investigation designed to determine the influence of the initial extension rate on subsequent survival during freezing.

Benson et al. (92) evaluated the pre- and post-freeze motility of spermatozoa subjected to initial extensions to 1,000, 250, 40, or 20×10^6 spermatozoa per milliliter in either yolk-citrate or yolk-Tris extender. The semen was then cooled in four hours to 5°C, at which time all samples were extended to a final concentration of 20×10^6 spermatozoa per milliliter. The highest pre-freeze

and immediate post-freeze motility occurred at an extension to 250×10^6 and a trend toward lower motilities at higher extension rates was observed. A slightly greater rate may be possible with yolk-Tris extender.

In a subsequent experiment these authors (92) compared the motility of spermatozoa in similar extension rates after 0, 4, 8, and 12 weeks of storage at −196°C. No pre-freeze dilution effect was observed, but statistically significant differences were found favoring lower extension rates in immediate post-thaw motility and motility after four weeks of storage. Samples stored for longer than four weeks were not influenced appreciably by the initial extension ratio.

17-4.2 Incubation. The fertility of bull semen that is used immediately after collection is not as high as that of semen that has been kept for a period of time (see Chapter 19) (93, 94). At 4 to 5°C the period is a day or less. then fertility declines (95, 96, 97, 98). However, further evidence that fertility improves after storage at 5°C has been provided by studies on equilibration and spermatozoal maturation. Post-thaw motility and/or fertility was improved by increasing the interval at 5°C between collection and freezing (74, 75, 78, 99–103). Furthermore, it has been shown that four hours' cooling time was significantly superior ($P < 0.05$) to one hour (92). The changes in spermatozoa that occur during equilibration may correspond to those responsible for the increase in fertility of liquid semen observed from the day of collection to the following day.

Elliott et al. (104) showed that although post-freeze motility was somewhat depressed, fertility was improved after mixing antibiotics with neat semen, followed by dilution and incubation at 30°C for 30 minutes before cooling. An improvement ($P < 0.01$) in fertility of two low-fertility bulls was recently reported as a result of incubation at 26°C for 20 minutes (106) irrespective of antibiotics. Thus it is possible that the improvement in fertility shown in these studies (104, 105, 106) resulted from cellular maturation and not entirely from the additional time provided for antibiotics to reduce the number and pathogenicity of organisms (107).

17-4.3 Cold Shock. Cold shock occurs when bovine spermatozoa are subjected to sudden cooling. The magnitude of the effect depends on the terminal temperature, the rate of temperature reduction, the extension ratio, and the length of time that spermatozoa are exposed to the terminal temperature (66, 115). The consequences of cold shock are irreversible loss of motility, increase in the number of spermatozoa that are permeable to vital stains, and significant reductions in respiratory activity and glycolysis (66, 115–119, 124–128).

Blackshaw and Salisbury (117) have shown a significant uptake of sodium and calcium and a loss of potassium and lipid phosphorus in cold-shocked spermatozoa. Quinn and White (120) also reported an increase in sodium and

calcium and a decrease in potassium and magnesium. It was further reported that dilution accentuated the cation movement of both cold-shocked and deep-frozen spermatozoa (120).

Other consequences of cold shock include coiled tails (121) and loss of lipid (19) and enzymes into the seminal plasma (122) (though a significant portion of glucose phosphate isomerase and lactic acid dehydrogenase are from protoplasmic droplets).

Epididymal spermatozoa are more resistant to cold shock than ejaculated sperm (123), and the loss of cold shock resistance seems to occur in the ampulla during ejaculation (124). Sperm in second ejaculates are more resistant to cold shock than sperm from first ejaculates (119), but this is probably true only when the interval between collections is longer than two days (125). Resistance to cold shock varies greatly among spermatozoa and is inherent in the individual spermatozoon (119, 123).

Cold shock can be prevented by cooling semen slowly in the presence of protective agents. The deleterious effects of cold shock are reduced by skim milk, casein, various milk extracts, and to some extent by bovine albumin, egg albumin, lecithin, egg yolk, and egg yolk extracts (116, 117, 118, 124). The protective mechanism must be one that stabilizes the cellular membrane thus reducing permeability. Dramatic changes take place in membrane permeability due to cold shock but more subtle changes may occur as well (126).

17-4.4 Cooling. It has generally been recommended that cooling of semen be commenced immediately after collection and extension, although, as stated above, an incubation period for raw or neat semen may be beneficial. In any event, semen should be added to an extender containing ingredients that will prevent cold shock and it should then be cooled slowly for maximum liveability of the cells.

VanDemark (108) and Foote and Bratton (109) have shown that the fertility of liquid semen was improved significantly ($P < 0.05$) by adding yolk-containing diluent to the semen before cooling. Despite this practice, rate of cooling can significantly affect fertility. Erickson (110) mixed semen and skim-milk extender at 32.2°C and then cooled split-samples to 3.3°C in either 110 minutes or four hours. Table 17-4 shows the fertility results of that study. The fertility of the samples cooled slowly (4 hours) was significantly ($P < 0.01$) higher than those cooled rapidly (1.8 hours).

In 1958, the National Association of Animal Breeders (NAAB) (111) found in a survey that, of the semen processors responding, 15 percent cooled semen in less than 1 hour; 52 percent in 1 to 1.5 hours; 10 percent in 1.5 to 2 hours; and only 23 percent allowed 2 or more hours for semen to cool.

Earlier laboratory experiments showed clearly that, for above-freezing storage, slow cooling was superior to rapid cooling in preserving motility and the capacity of sperm to metabolize substrates (112, 113, 114). An interaction was shown between temperature and cooling rate, indicating that a slower

Table 17-4. Effect on fertility, expressed as 30–40-day nonreturns,[a] of two rates of cooling bull semen in heated skim milk plus antibiotics.

Variable	*First services*	*% NR*
Skim milk + antibiotics (cooled rapidly)	1,772	74.5
Skim milk + antibiotics (cooled slowly)	1,817	80.6
Skim milk + antibiotics + 10% glycerol (cooled slowly)	1,759	81.5

[a]From Erickson (110). Unpublished results (1958) used by permission.

cooling rate was required for storage at 1°C than at 5°C (112). Similar results have been obtained (48, 92) for frozen semen. Post-thaw motility was maintained significantly longer in semen that was cooled 4 hours before freezing than in semen cooled only 1 hour (92). Similarly, use of live-dead staining techniques have shown that longer cooling times preserve the life of spermatozoa better than faster ones.

To our knowledge, no fertility studies have been conducted with frozen semen using cooling rate as a variable. The desire to complete semen processing and freezing on the day of collection seems to have resulted in a cooling rate of convenience rather than one based on sound experimental data. Although sufficient data are lacking for specific and precise recommendations, there is considerable danger in rushing the cooling process.

Figure 17-5 shows the equipment used in the laboratory at Colorado State University to obtain a slow (4 hours) cooling rate. It consists of a 600-milliliter nalgene beaker and a circular piece of masonite, 4½ inches in diameter, to which is glued a piece of cork ¾ inch thick and 3¼ inches in diameter. A hole 1 inch in diameter is drilled through the masonite and cork. Tubes of several sizes are fitted with rubber rings to suspend the tube in the 600-milliliter beaker filled with 38°C water. Tube size varies to permit selection of a tube most suited for the volume of semen and extender.

A typical cooling rate, measured in the center of the test tube with a copper-constantin thermocouple, is presented in Figure 17-6. The rate of temperature reduction can be accelerated at any point along the curve by removing the tube and placing it in a test tube rack in the cold room.

Every AI unit should own and use a suitable, direct-reading instrument for measuring temperature with wire thermocouples (see Figure 17-7). The expense is minimal and could prevent some disastrous results. By placing the small thermocouple in the center of the diluted semen and conducting a few simple trial-and-error experiments, the desired cooling rate can be established for any set of conditions. Furthermore, the temperatures of the refrigerators,

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17-4.5 Glycerol Ad

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Figure 17-5.

various locations within cold rooms, and waterbaths should be checked at frequent intervals.

17-4.5 Glycerol Addition.

Temperature of Glycerol Addition. Polge (127) reported that spermatozoan revival after freezing was greater when glycerol was added at 5°C than when it was added at 20°C. Miller and VanDemark (43) obtained a slightly higher percentage of motile spermatozoa when glycerol was added at 4.5°C than at 10°C and both of these were superior to addition at 15.5°C ($P < 0.01$). Others have concluded that spermatozoa were damaged upon exposure to glycerol at temperatures above 5°C (53, 128, 129) and found that the damage to

Figure 17-5. Equipment that will permit slow cooling of semen.

spermatozoa exposed to glycerol at 32°C was more pronounced as the length of glycerol exposure at this temperature increased (130).

Other investigators (76, 131, 132) have not observed a depression in post-thaw motility following glycerolization at temperatures above 5°C. In one investigation there were no differences in 75-day NRs for spermatozoa glycerolated at 5, 10, 20, or 35°C (132).

The damaging effects of exposure to glycerol at higher temperatures are at least partially reversible upon subsequent equilibration at 5°C (43, 130). It is likely that survival would be most severely depressed in samples glycerolated at higher temperatures when equilibration time was also shortened.

Loss of the enzyme glutamic-oxalacetic-transaminase (GOT) was greater from bull spermatozoa glycerolated in yolk-citrate at 37°C than at 5°C. The rate of release was no different after 4 hours of equilibration at those temperatures (133). With a Tris-yolk extender and 3 hours' equilibration there was no depression of fertility by addition of the glycerol at 35°C (78).

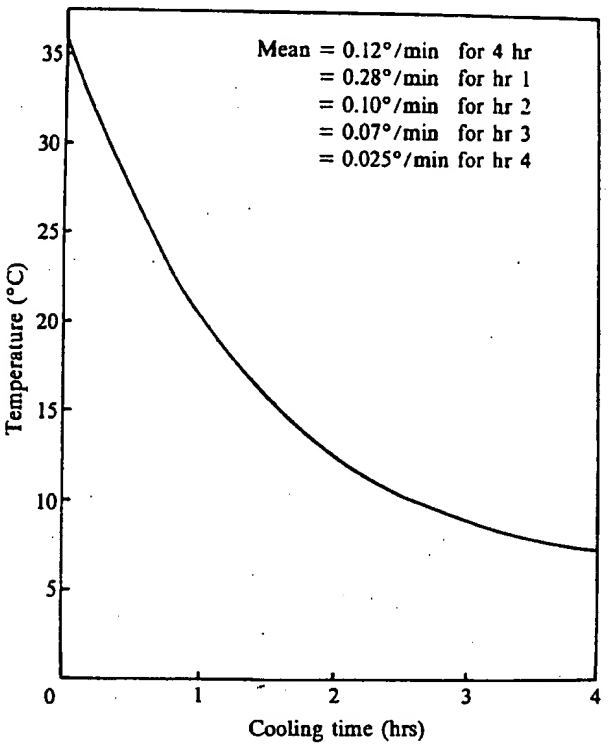

Figure 17-6. A typical semen cooling rate at the center of the test tube depicted in Figure 17-5.

Rate of Glycerol Addition. Post-thaw motilities of 38 and 51 percent were observed when glycerol was added in 1 or in 3 steps at 20-minute intervals (53), respectively. In a subsequent experiment no significant differences were found between 4 and 16 equally spaced glycerol additions during a 1- or 2-hour period. An advantage of stepwise addition of glycerol to bull semen was observed, but no more than 4 additions were necessary.

In contrast, no differences were found in the post-thaw motility of spermatozoa glycerolated at 5°C in 5 equal additions at 6- or 12-minute intervals, in 3 equal additions at 10-minute intervals, in a single addition (134) or an experiment with glycerol addition in 1, 3, or 5 equal portions (43). The fertility of spermatozoa frozen after glycerolization at 5°C in either 1 or 4 steps immediately after cooling was compared with 1 step 5 hours after cooling (1 hour

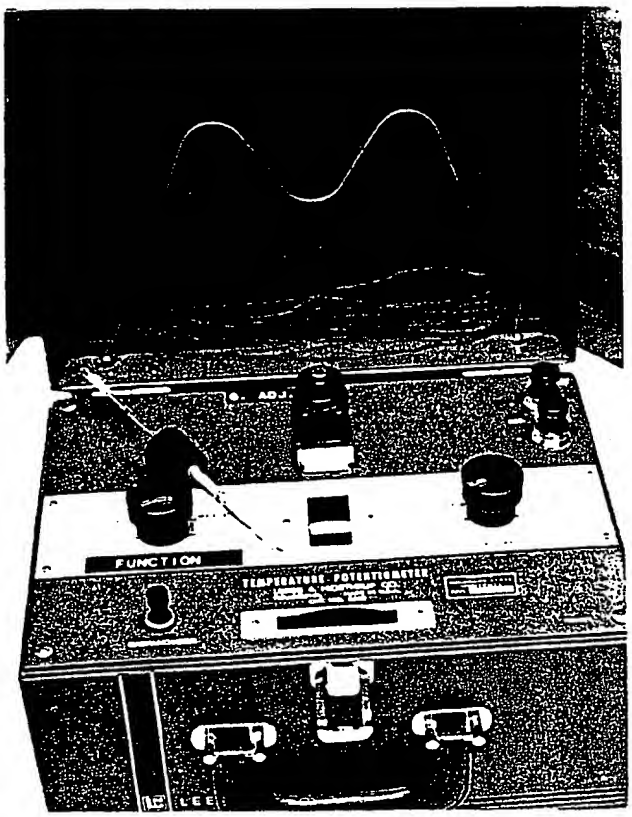

Figure 17-7. A potentiometer capable of measuring temperature by means of wire thermocouples.

before freezing). Fertility was not significantly influenced by the method of glycerol addition; it was similar for semen frozen in straws when samples were glycerolated either slowly, over 2 hours, or quickly (135).

Under some conditions the stepwise addition of glycerol to bull semen may enhance post-thaw motility; the advantage has generally been small, however, and may not be reflected in increased fertility (78, 135).

It has also been reported (136) that the liveability of spermatozoa in liquid semen was enhanced by adding glycerol stepwise in 20, 30, and 50 percent portions at 10-minute intervals, as compared to addition in a single step. Dropwise glycerolization has been employed successfully for both liquid (136) and frozen semen programs.

Shannon in New Zealand (137) has described a novel approach to the bulk freezing of semen that may be applicable to the seasonal breeding of beef cattle in the United States (146). Semen was initially diluted about 1:1 for cooling to 5°C, followed by dilution to a concentration of 600×10^6 sperm/ml without glycerol. The extended semen was then placed in dialysis tubing about 5 inches long and 0.25 inches in diameter, and suspended in extender that contained glycerol. This allowed the exchange of glycerol and metabolic end-products. Following a period of equilibration, the material in the dialysis sack was frozen. By freezing semen in bulk at a high sperm concentration, efficient utilization of labor and storage space was achieved. This technique was well suited to the seasonal breeding of dairy cattle in New Zealand.

17-4.6 Equilibration. Investigators (35, 74, 75, 99-103, 138) have demonstrated that post-thaw motility and fertility of bovine spermatozoa are both enhanced by extending the interval between adding the glycerol and beginning freezing. This stage of semen processing has been called the "glycerol equilibration" period. The results of several fertility trials designed to determine the optimal glycerol equilibration period have been summarized in Table 17-5. These studies clearly established the beneficial influence on the fertility of bull spermatozoa of an equilibration period of several hours.

Experiments have been conducted in which equilibration times have been varied at 5°C both in the presence of glycerol and without glycerol (53, 78, 101, 139, 140). The goal has been to determine whether it is spermatozoan-glycerol interaction or a process of sperm-cell maturation. A number of investigators (35, 75, 78, 138) have found evidence that lengthy glycerol equilibration per se is not as essential as the time in storage at 5°C before freezing. Jondet's (141) experiments using straws as containers for frozen semen suggest the same conclusion.

It is clear that additional fertility trials with the different methods of freezing are desirable, but on the basis of existing information 4 to 18 hours of storage at 5°C before freezing are recommended; and it appears that glycerol can be added at any time during this period without affecting fertility.

Table 17-5. The influence of glycerol equilibration on non-return rates.

Equilibration time	*No. services*	*% NR*	*Ref.*
0.5 hr	449	56.2[a]	(99)
18 hr	450	63.9	
30–50 min	334	71.8[b]	(100)
overnight	330	78.1	
0.5–1.0 hr	864	54.4[c]	(101)
18 hr	864	61.4	
4 hr	996	63.4[d]	(102)
8 hr	1,026	65.2	
12 hr	1,012	67.8	
2 hr	2,256	66.5[e]	(103)
18 hr	2,176	71.3	

[a]30-day NR.
[b]31-day NR.
[c]3-month NR.
[d]75-day NR.
[e]60–90-day NR.

17-4.7 Sperm Numbers. The demand for semen from genetically superior sires has made it necessary that a minimum number of frozen sperm cells be established for maximum fertility of each insemination (142–154). Fertility is the single most important consideration in AI and no fewer sperm should be used than are necessary. One problem in recommending a minimum number is that new extenders and new methods of processing, freezing, and packaging make continual reevaluations necessary.

Table 17-6 shows the influence that the number of spermatozoa per insemination has on the fertility of semen frozen in 1.0-milliliter ampules. Since only one study (231) resulted in statistically significant differences, the lowest number of spermatozoa could logically be selected in all except that study. In all except one of the seven studies, however, mean fertility was higher with the highest number. Thus for maximum reproductive efficiency, the higher numbers of spermatozoa presented in Table 17-6 should be used.

Experiments illustrated in Figures 17-8 and 17-9 (151, 186) show that the number of spermatozoa per insemination is somewhat higher for the bull with

lower than average fertility. Thus the fertility level of each bull in a breeding operation must be considered before the absolute number of spermatozoa for each insemination can be established.

Table 17-7 summarizes the results of experiments on the number of spermatozoa necessary in extended semen that is to be packaged and distributed in straws (135, 147, 148, 149, 153, 189, 232–234, 235). These studies have also shown differences among bulls. In another study (153) the fertility difference between "good" and "poor" inseminators decreased as sperm numbers increased.

17-4.8 Packaging: Ampules, Straws, Pellets. At first, bull semen was packaged for freezing in glass ampules. Then a process for freezing extended semen in straws was developed, and more recently semen has been frozen in pellets. Satisfactory conception rates have been reported with all three systems (247, 248).

Danish investigators developed the idea of packaging and freezing extended semen in straws and were also responsible for much of the other earlier work. Soda straws were used in the first studies. French workers Cassou (156) and Jondet (157) have refined the technique. The French straws are plastic containers 113 millimeters long. They vary in diameter from 2.0 millimeters (0.25 milliliter) to 4.2 millimeters (1.2 milliliters), with an intermediate diameter of 2.8 millimeters (0.5 milliliter). More recently, groups in Canada, West Germany, and the United States have developed systems using straws of various sizes (see Figure 17-10). Macpherson and Penner (155) have reviewed the history of this technique and have described a modified method developed in Canada.

In the French and Canadian techniques, one end of the straw is plugged with a powder of polyvinyl alcohol that forms a seal when it comes into contact with an aqueous solution, the semen. The other end is ultrasonically or electrostatically sealed after filling. West German and U.S. straws are sealed with nonabsorbable plugs (see Figure 17-10).

The system has now been automated and its popularity is likely to increase. The straw system requires less cold storage space: Field units will accommodate at least twice as many straws as ampules; and central storage units will hold three to five times as many (158). There might also be some advantage with regard to fertility (see Table 17-8) (247, 248).

The technique of freezing semen in pellets was originally developed by Japanese workers (44, 163) and has not been widely adopted by the AI industry in the United States or Western Europe. The system has not been automated, and permanent labeling of the pellets has proven difficult. Furthermore, there is the possibility of contamination (248) by bacteria in the process. To freeze semen by the pellet method, small depressions are made in a block of solid CO_2 to accommodate the desired volume of semen. Volumes of 0.05 to 0.2 milliliter are placed in the depressions on the dry ice (see Figure 7-11), where the semen is allowed to remain for about 10 minutes before transfer to liquid nitrogen storage.

Table 17-6. The influence of number of spermatozoa per insemination on the fertility of semen frozen in 1.0-milliliter ampules.

Spermatozoa (10^6)	*Inseminations*	*% NR*	*Ref.*
13.3[a]	490	61.0[b]	(84)
20.0	490	63.3	
30.0	489	61.5	
20.0[c]	5,607	71.7[d]	(146)
30.0	5,638	72.2	
16.0[c]	1,977	72.8[d]	(78)
24.0	2,103	74.6	
16.0[c]	5,916	73.1[d]	(78)
24.0	5,831	73.2	
12.0[c]	13,939	70.4[d]	(78)
24.0	13,060	71.6	
12.0[a]	—[e]	70.6[d]	(151)
24.0	—	72.0	
35.0	—	73.1	
10.0[c]	1,576	61.1[d]	(231)
15.0	1,488	61.5	
20.0	1,539	69.5	

[a]Total spermatozoa.

[b]3-month.

[c]Motile spermatozoa before freezing.

[d]60-90-day.

[e]20,815 cows were utilized for the experiment.

Table 17-7. Influence of number of spermatozoa per insemination on the fertility of semen frozen in straws.

Spermatozoa (10^6)	*Inseminations*	*% NR*	*Ref.*
10[a]	557	61.6	(232)
20	527	61.9	
30	565	65.7	
5[a]	624	55.6	(232)
10	622	58.7	
20	638	61.0	
10[a]	565	79.5	(147)
20	527	78.6	
30	557	78.6	
5[a]	532	76.5	(147)
10	533	74.5	
20	550	77.3	
20[a]	2,665	58.4	(233)
30	2,888	60.2	
8[b]	2,648	69.7	(149)
12	2,551	73.3	
.375[c]	805	49.9	(148)
.75	816	57.4	
3	765	65.8	
14	836	71.8	
5[a]	993	74.0	(234)
10	998	79.5	
15	990	76.7	
20	995	79.2	

[a]Total spermatozoa.

[b]Motile spermatozoa before freezing.

[c]Live spermatozoa after thawing.

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17-5 FREEZING AI

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Spermatozoan concentrations in the pellets are about 10 times higher per milliliter of extended semen than in ampules, and the pellets therefore use far less storage space. Pellets are thawed rapidly in enough warm extender (163) to provide the right volume and the proper number of spermatozoa for each insemination.

The choice of packaging system should depend upon conception rate, economics, and convenience, in that order.

17-5 FREEZING AND THAWING

17-5.1 Freezing Rates. In early studies, ampules of semen were frozen in a dry ice-alcohol bath. Cooling rates of 0.8 to 3°C per minute from 5 to −15°C, and 3 to 5°C per minute from −15 to −79°C, were maintained by controlling the rate at which dry ice was added to the bath. Slow freezing rates were considered essential until Luyet and Keane (164) froze semen by transferring

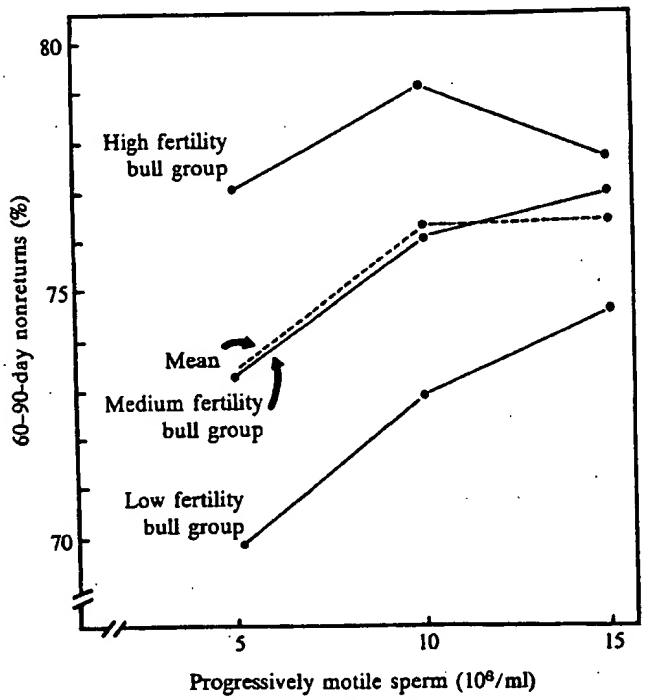

Figure 17-8. Nonreturn rate as affected by motile spermatozoan concentrations and fertility level of Holstein bulls. [From Sullivan. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 36–43. 1970.]

ampules abruptly from 0°C to an alcohol bath at temperatures from −20 to −50°C, where it was held for five minutes and then transferred abruptly to liquid nitrogen. When the intermediate temperature was −20°C virtually none of the cells survived freezing. In contrast, an intermediate temperature of −27°C provided a high rate of survival. Thus the existence was established of a critical temperature range in which rapid cooling was detrimental to spermatozoan survival. This finding was supported by a number of investigations in which conventional freezing rates were employed (165, 166) and was consistent with the report (167) of an optimal cooling rate of 4 to 20°C per minute between −10 and −30°C (see Figure 17-12).

In contrast, other workers (168) found that semen could be transferred directly to the bath at −20 and −40°C for subsequent freezing at the normal

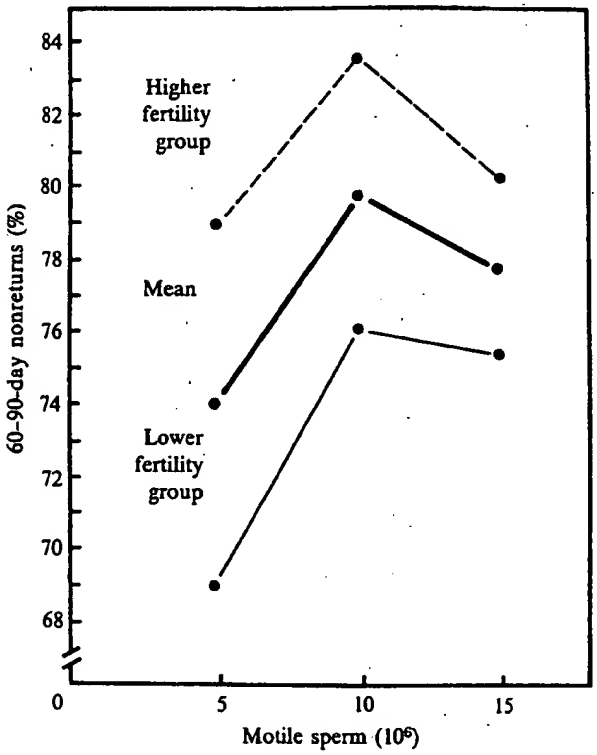

Figure 17-9. Nonreturn rate of dairy cows as affected by motile spermatozoa concentration and fertility level of semen from Angus and Hereford bulls. [From Sullivan. Proc. 3rd Tech. Conf. Artif. Insem. and Reprod. NAAB. pp. 36–43. 1970.]

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rate. It was apparent that spermatozoa could be cooled rapidly between 5°C and −40°C. This was supported by the successful freezing of spermatozoa in pellets (32, 163), films (169), and by direct placement into liquid nitrogen (32). With these techniques spermatozoa are cooled rapidly from 5°C to −79° or −196°C (see Figure 17-13) (170). Spermatozoa may be frozen successfully using a variety of rates and methods.

The inconvenience of manually regulating the cooling rate has led to the evolution of programmed instrumentation techniques and then simplification to the point where the semen is now frozen in the nitrogen vapor of a liquid nitrogen container (171–174). These techniques are simple and convenient and they eliminate the need for costly automation. The freezing rate in such a system depends upon the ampule's position within the nitrogen vapor. Typical freezing curves for ampules frozen in liquid nitrogen vapor are presented in Figure 17-14. The comparable survival rates of spermatozoa in ampules that occupy different positions within the vapor supports the contention that spermatozoa can withstand a wide range of freezing rates.

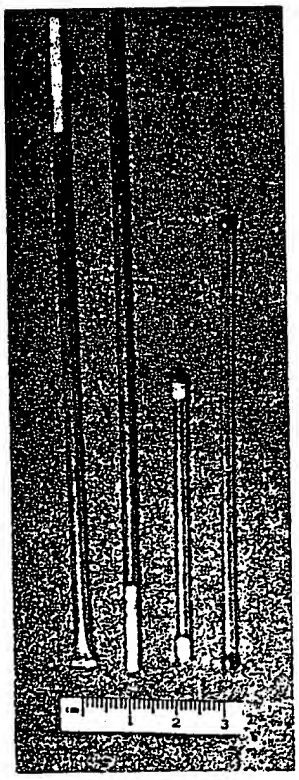

Figure 17-10. Various types and sizes of straws currently in use. From left to right: Canadian, French, U.S., and German straws.

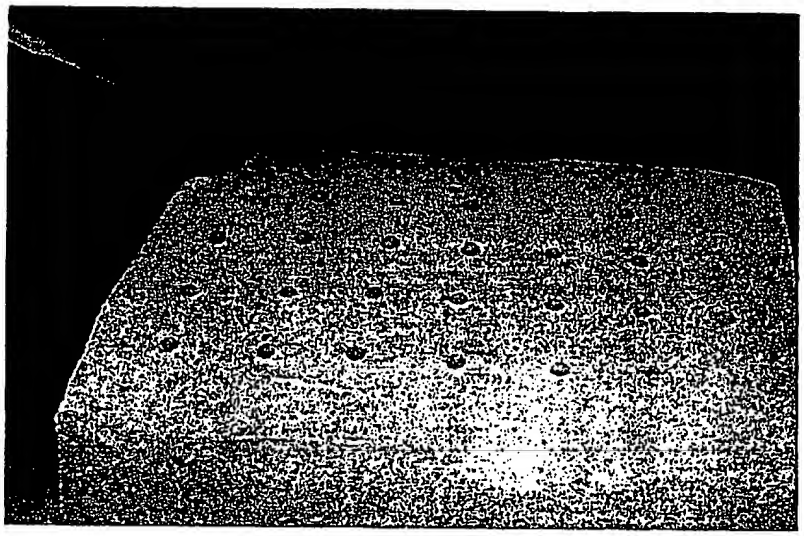

Figure 17-11. Semen being frozen by the pellet method.

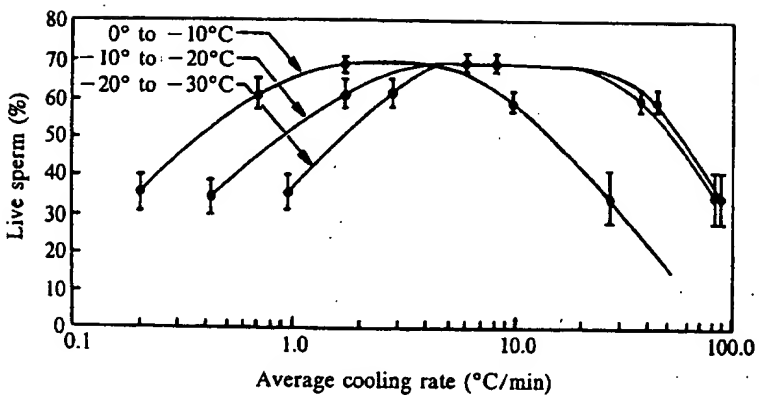

Figure 17-12. The optimum range of cooling rates between −10° and −30°C. [From Bruemmer et al. *J. Cell and Comp. Physiol.* 62:113–118. 1963.]

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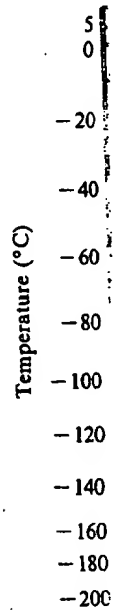

Figure 17-13. C
left to right repre
at −79°, −100°
Salamon. *Aust. J*

If the current popularity of the straw method of packaging continues, the use of vapor freezing methods will become more extensive.

17-5.2 Storage Time and Temperature. The cost of storing material at temperatures below freezing increases as the temperature decreases. Thus for economical reasons it is necessary to determine the temperature that will provide extended storage, minimum expense, and minimum deterioration in spermatozoan quality. VanDemark et al. (37) have shown (see Figure 17-15) that storage temperature has a dramatic effect on sperm motility after storage.

A number of detailed experiments have been conducted to elucidate the details of the effects of storage temperature and duration on the survival and fertility of frozen bull spermatozoa. The results show not only the effects of the main factors already discussed but important interactions among these factors. In general the data indicate that the lower the storage temperature the longer motility and fertility can be maintained. To ensure this, however, it is

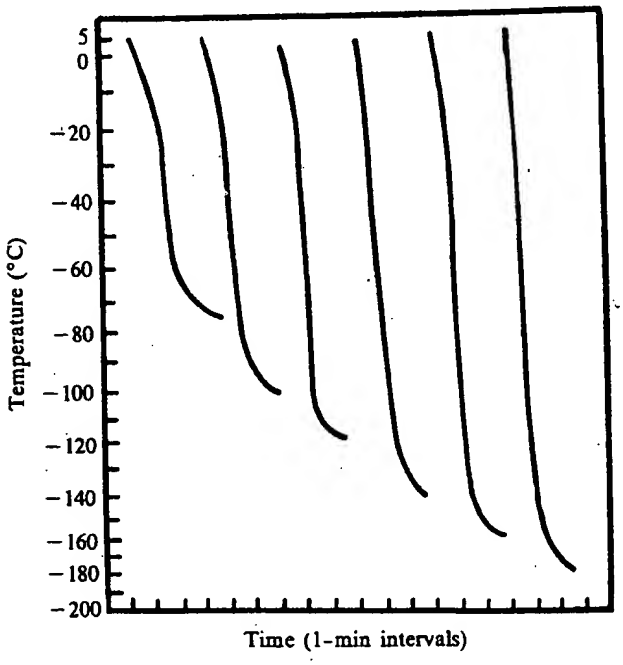

Figure 17-13. Cooling rates at the center of 0.3-milliliter pellets. Curves from left to right represent freezing on dry ice (−79°C), and on a stainless steel plate at −79°, −100°, −110°, −140°, −160°, and −180°C, respectively. [From Salamon. *Aust. J. Biol. Sci.* 23:459–468. 1970.]

necessary to use the procedures, timing sequence, and other techniques that are optimum for that lower holding temperature. In one experiment (175) using split ejaculate samples, the motility of the spermatozoa in semen frozen to −79°C declined more rapidly during incubation after thawing than the spermatozoa in semen that had not been frozen. When a thrice-repeated freezing-thawing stress was applied to semen when it was first frozen to either −79°C or −196°C no differences were observed in the resistance of the contained spermatozoa to this stress at either temperature. When the semen was stored for 6 months at these temperatures, however, the spermatozoa kept at −196°C withstood the repeated stress better than those kept at −79°C (176). Similarly, when semen was exposed to ambient room temperature for short intervals after 0 to 120 days' storage, spermatozoa in liquid nitrogen were more resistant than those from the same ejaculate frozen in dry ice/alcohol (177). Greater deterioration in motility and metabolic activity of spermatozoa occurs at −79°C storage than at −196°C regardless of the storage interval (178, 179, 180).

Semen transferred from −196°C survived better on subsequent storage at −79°C after holding at −196°C for 7 or 10 days rather than for 3 days (182).

Investigations involving temperatures of −79, −92, −96, and −196°C show an advantage for temperatures below −79°C (80, 181) for spermatozoan survival and motility recovery. Spermatozoa in milk extenders, however, appear to require a lower holding temperature than yolk-citrate extender for optimum motility recovery after several months of storage (80)

Some investigators have reported a gradual improvement in the post-thaw recovery of motility after 3 weeks' storage at −196°C (179) and for as long as 24 weeks at −96°C, suggesting that, over time, spermatozoa make a significant adjustment to the conditions of storage (181). More recent studies (183, 184) show that fertility increases for a time immediately after freezing. A similar increase has been observed at the beginning of storage at temperatures above freezing. Whether the fertility of frozen semen in commercial AI will increase with storage depends largely on the procedures used in processing and freezing. Fertility is not likely to increase if, for example, the semen has been subjected to a relatively long incubation or equilibration after cooling immediately upon collection and before freezing.

Studies on the effects of temperature on rates of chemical change and the effects of storage indicate that fertility will be maintained longer at the lowest practical storage temperature (−196°C) than at higher temperatures. Considering the expected temperature control of metabolic turnover from body temperature to −196°, no system of semen storage has produced results comparable to those in which rabbit spermatozoa in the ligated epididymis were held in the scrotum at a few degrees below body temperature (see Section 8-7.4). Based on a temperature coefficient of 2, which would halve the metabolic activity each drop of 10°C the 38-day period found by Hammond and

Asdell (251) for ferti
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Table 17-8. Effe

	Straws
% NR	*Inse*
67.2	
73.0	
70.4	
70.7	
63.7	
71.1	
72.0	
67.7	
67.5	
61.1	
69.0	
71.0	
74.3	
71.5	
73.2[a]	
70.7	
74.9	

[a]Percent pregnant by
[b]First and repeat ser

Asdell (251) for fertility maintenance in the ligated rabbit epididymis would be comparable to a hundred years or so at −196°C. Theoretically, optimum fertility in cattle spermatozoa can be maintained far longer at −196°C than it has ever been.

Mixner (154) has shown, with a limited number of services (626 in the 12 years), that bull spermatozoa remain fertile when stored at −79°C for 8 years and at −196°C after that for up to 12 years. After performing similar long term storage tests with a total of 1310 inseminations Stewart (185) has reported a conception rate of 58 percent after 9 years. This represents an average yearly decrease of 0.8 percent NR. The report by Salisbury (183, 186) for bull semen stored in glass ampules at −79 to −88°C refers to all semen used by one AI

Table 17-8. Effect on fertility of packaging semen in straws or ampules.

Packaging system				
Straws		*Ampules*		
% NR	*Inseminations*	*% NR*	*Inseminations*	*Ref.*
67.2	–	64.3	–	(236)
73.0	78	73.9	69	(237)
70.4	1,462	67.7	1,909	(161)
70.7	888	73.8	725	(238)
63.7	–	57.9	–	(239)
71.1	305	69.5	240	(159)
72.0	150	67.8	158	(240)
67.7	176,293	69.1	161,151	(241)
67.5	7,380	67.6	7,188	(160)
61.1	3,915	65.5	3,847	(160)
69.0	–	67.5	–	(242)
71.0	599	67.0	625	(243)
74.3	354	73.2	381	(243)
71.5	5,544	70.3	5,637	(243)
73.2[a]	395[b]	54.3[a]	383[b]	(244)
70.7	1,407	72.7	1,448	(245)
74.9	7,592	72.8	6,714	(162)

[a]Percent pregnant by rectal palpation.

[b]First and repeat services.

organization for inseminations during 1962 and for all semen collected in 1962 and used either in that year or in 1963. A total of 216,222 first service inseminations were involved. For a major organization at that time it was a very precise study of fertility expectation and of seasonal influences on fertility; but it was a less precise estimate of the potential duration of fertility for selected semen. Fertility peaked after 2 months of storage at −79°C, and stayed at a maximum for about 4 months, after which it decreased at the rate of 5 to 6 percent NR a month. When semen is transferred from liquid nitrogen to dry ice-alcohol the ability of the spermatozoa to recover motility is depressed (178, 179, 182). This ability lasts longer in liquid nitrogen (80, 176, 177, 178, 180, 182). Direct comparisons of fertility after storage at the two temperatures for less than 6 months are slightly in favor of the lower temperature (145, 182, 185) (see Table 17-9), but no long term comparisons have been made of fertility duration using samples of the same semen stored at the two temperatures. Salisbury's study (183) dealt with the fertility level found for the same AI unit after a shift to semen freezing and storage under liquid nitrogen by

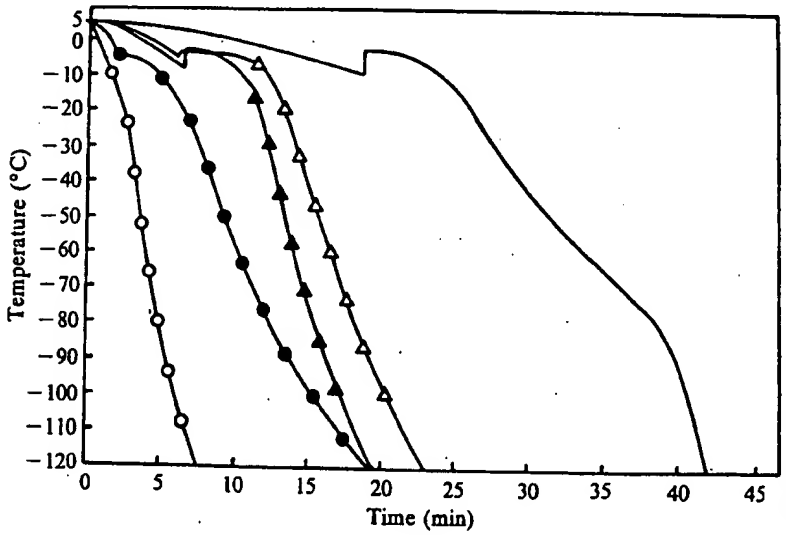

Figure 17-14. Representative intra-ampule cooling rates for semen frozen in an automatic freezer and in liquid nitrogen vapor. The various lines represent the following: ——, control (automatic freezer); △, top ampule in center of cardboard box (vapor frozen); ▲, third ampule from top in center of box (vapor frozen); ●, third ampule from top in outside corner of box (vapor frozen); and ○, bottom ampule in center of box (vapor frozen). [From Clegg et al. Univ. of Conn. (Storrs) Agr. Exp. Sta. Res. Rep. 5. 1965.]

the inseminator technicians with weekly shipments out of central storage. The results for 196,448 first service inseminations show an increase in fertility for the first 4 months of storage at −196°C, after which fertility peaked for the next 8 months and then decreased during the second year of storage. Clegg and Pickett (187) studied 21,488 inseminations with semen stored at −196°C. They found no decrease in fertility over 2 years of storage. Foote (191), who obtained the same results, also found no evidence of important seasonal variations in fertility such as those that had been shown in the Illinois studies (183, 186). Fertility may last longer under liquid nitrogen if the storage conditions, including the actual semen temperature, are kept constant.

In studying semen stored in straws at −196°C Cassou (189) found no differences in fertility between 1 and 3 months (69.8 percent NR on 9,053 first services); between 4 and 6 months (68.4 percent NR on 11,472 first services); or between 7 and 66 months (69.1 percent NR on 83,843 first services). Ström (188) made a similar study of semen frozen in pellets and held in liquid nitrogen. A total of 63,863 cows were inseminated. He found no evidence of a change in fertility over a mean storage period of 44 weeks.

The latest data available on the questions raised by these studies are reported in a recently completed statistical analysis at the Illinois Agricultural Experiment Station (252). This analysis was performed on data provided by

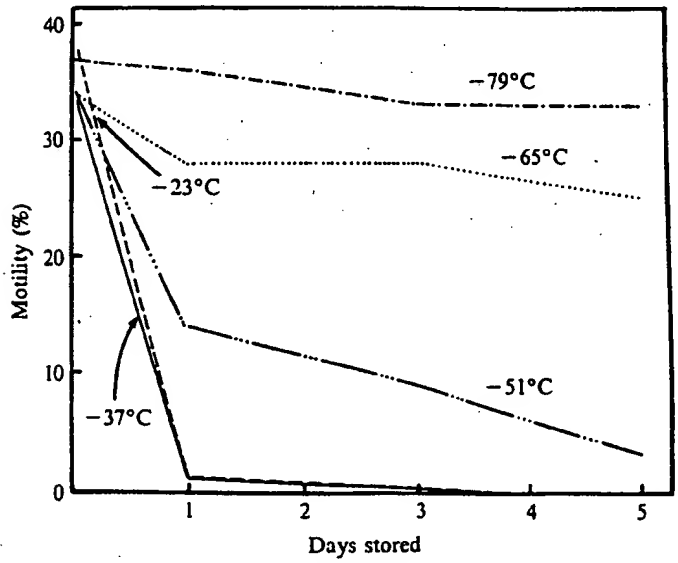

Figure 17-15. The effect of storage temperature upon post-thaw motility. [VanDemark et al. Illinois Agr. Exp. Sta. Bull. 621. 1957.]

Table 17-9. Effect of storage temperature on nonreturn rate.

Storage temperature					
−79°C		−196°C			
First services	*% NR*	*First services*	*% NR*	*Difference*	*Ref.*
463	69.3	495	71.9	2.6	(182)[a]
1,300	58.4	1,251	60.1	1.7	(185)[b]
4,791	68.4	4,951	71.1	2.7[d]	(144)[c]

[a]75-day NR.
[b]112-day NR.
[c]60-90-day NR.
[d]$P < 0.01$.

the Michigan Animal Breeders Cooperative on 555,449 first service inseminations made with semen stored in ampules at −196°C from 1965 to 1970. Some of the semen was used 5 years after collection. The day of seminal collection and the day of insemination were recorded for all inseminations. The data show the fertility responses to length of storage were significantly different among ejaculates both from different bulls and from the same bull. Fertility increased with time after collection and freezing (the use of semen on 5th to 15th day after freezing tended to yield the lowest 180-day NR rate). In one separate analysis of 97,586 inseminations using semen from 123 bulls maximum fertility (180-day NR) occurred after 810 days or about 2.2 years of storage. In a 2-way analysis of variance of 108,311 inseminations using semen from 133 bulls maximum fertility occurred when semen had been stored 1260 days or about 3.4 years. In neither case was there a statistically significant decrease in fertility with time up to 1260 days, though the variability did increase greatly. In each case, however, these samples were selected to test, as well as possible, the duration of fertility under nitrogen storage. When a regression analysis was calculated, using semen one year old or less and involving 83,198 inseminations from the semen of 122 bulls, the maximum fertility occurred for semen that was 255 days old. This fertility level stabilized for a time and then declined. These analyses indicate that, with proper selection of bulls and ejaculates and proper handling, some semen can maintain optimum fertility for relatively long periods—up to 3.4 years in one study.

17-5.3 Handling Techniques. Until recently, most cows in commercial AI programs were bred by full-time, closely supervised technicians. The sale of semen directly to the herd owner, with insemination to be performed by him

or by a farm employee, has posed an entirely new set of problems. This is particularly true with the AI of beef cattle, in which an inseminator often breeds a large number of animals on a seasonal basis during a short period of time. This often requires an annual relearning of skills, often with little or no supervision. It is therefore important for the inseminator to review the proper methods of semen handling periodically throughout the year (190). Many of the fertility problems associated with the use of frozen semen are due to improper handling or deposition of the semen.

In many cases, the first mistake comes when the semen is received at the farm or ranch and must be transferred from one storage unit to another. This should take place out of the wind and sunlight. Wind increases the rate at which the temperature of the ampule rises by removing the "layer of cold" that surrounds the ampule. The effect of sunlight on frozen semen is not known, but it is not likely to be beneficial and it could actually be harmful.

Frozen semen is further exposed to elevated temperatures when the semen is removed from storage tanks, when they are opened as inventories are taken, etc. The amount the temperature rises is determined by:

1. length of time exposed,
2. ambient temperature,
3. air circulation,
4. intensity of solar radiation,
5. nitrogen level in the tank, and
6. the height to which the canister is raised above the liquid nitrogen level.

When semen is transferred the exchange should be made quickly into tanks previously filled with nitrogen. It is essential that semen is handled properly, which involves careful temperature control more than anything else, in order to prevent fertility reduction. It is reasonably clear from the work reported by Sullivan (150) (see Figure 17-16), that, as sperm numbers are reduced to obtain maximum use of each superior bull, the storage temperature, exposure times, and so on will become more critical. It has been shown that exposing frozen semen in the neck of a liquid nitrogen tank generated no significant decrease in motility unless the exposure was relatively long (192). The fertility of fluid semen, however, decreases much more rapidly than motility (79) and the same is probably true of frozen semen. A fairly high correlation has been shown (177) between fertility and loss of motility due to exposure of ampules of frozen semen to ambient temperatures. Thus it is extremely important to minimize the number and times of exposures (see Figure 17-17). It is much more harmful to expose ampules of semen to elevated temperatures uninterruptedly for one minute than to expose them for two minutes in 10-second periods, particularly if the ampules are returned to $-196°C$ between the 10-second exposures.

17-5.4 Thawing. It is difficult, if not impossible, to separate the effects of thawing from those of freezing. Theoretically, the faster a specimen is frozen the more rapidly it should be thawed for optimal survival (193). Furthermore, the results obtained from thawing studies may be influenced by other events (43, 128, 167, 193, 194). Interrelationships have been shown between thawing and freezing rates (167) and extenders (128) and other factors, such as glycerol concentrations, may contribute to some of the discrepancies in the literature (194).

Rate of thawing significantly affects the survival of spermatozoa that have been frozen (43, 128, 134, 159, 167, 194). The rate at which thawing occurs depends on the size and shape of the container (ampule, pellet, straw) in which the semen is stored, the composition of the container (plastic, glass), the thawing medium (water, air), the temperature imposed, and the type of thawing system (195).

Thawing in Glass Ampules. It has been reported that thawing bovine semen in ampules in water maintained at 38 to 40°C resulted in superior sperm survival

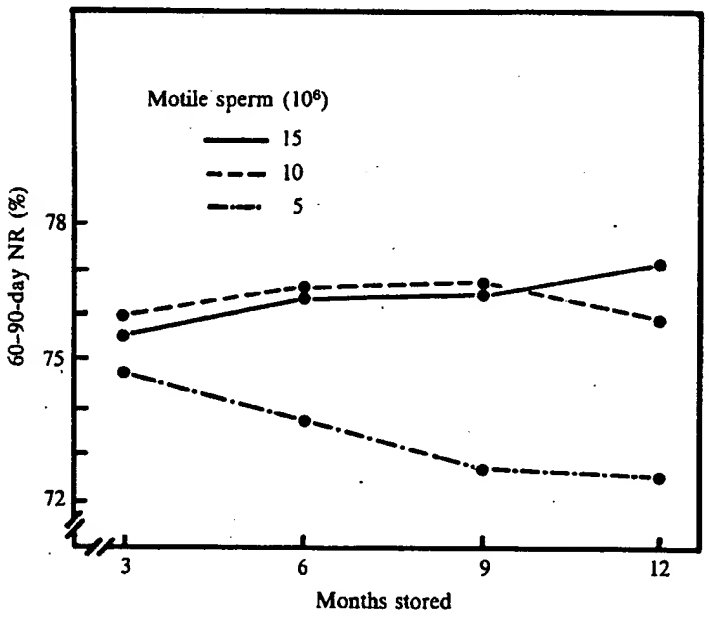

Figure 17-16. The influence of sperm numbers on the fertility of semen stored for up to one year. [Sullivan. Proc. 3rd NAAB Tech. Conf. Artif. Insem. and Reprod. pp. 36–43. 1970.]

as compared to thawing at lower temperatures (128, 167). Miller and Van-Demark (43), however, using 5-milliliter plastic ampules containing 1 milliliter of extended semen, observed significantly greater motility in samples thawed at 5°C than in those thawed at 38°C; others (134, 194, 196, 197) found no difference in survival between spermatozoa water-thawed at 38 to 40°C and those thawed at 5°C. In general, thawing in air or water at intermediate temperatures of 15 to 22°C resulted in decreased survival (134, 142, 194, 198). It is exceedingly difficult to explain why an intermediate thawing rate is inferior to a slower rate, since it has been postulated (127, 128) that thawing time between −15 and −30°C should be as rapid as possible. Although this may be expected on a theoretical basis, the critical range for freezing may not correspond to a critical range for thawing.

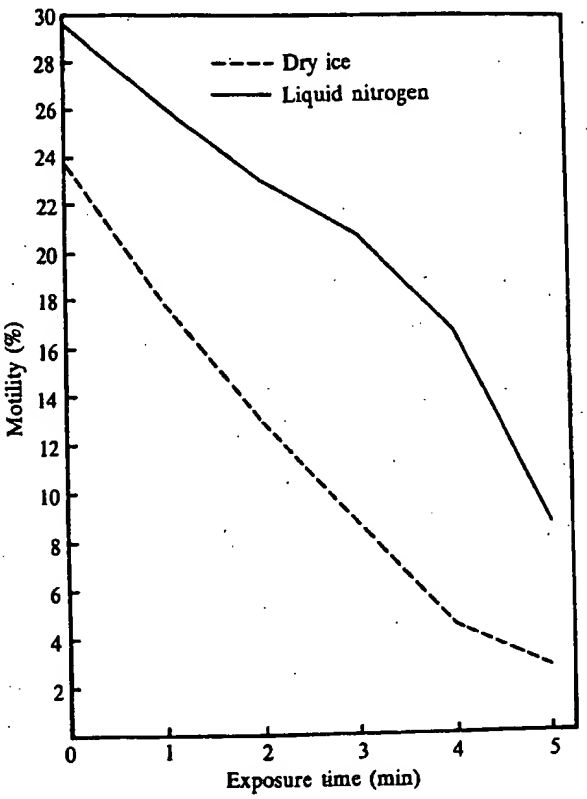

Figure 17-17. The effect of exposing ampules of frozen semen to ambient temperature on percent motility. [From Pickett. *A.I. Digest* 19(2):8–23. 1971.]

The end fertility level is the ultimate test of superiority of any semen handling method. Unfortunately such data on thawing rates are limited. Arnott (142) conducted two studies comparing semen thawed in water at 15 and 30°C, and at 4 and 15°C. A significant difference in 60–90-day percent NR was found in favor of 15°C in the first study, and a 2.0 percent NR difference in favor of thawing at 4°C in the latter study. These latter results agree with those of Pickett et al. (194), who found a difference of 1.26 percentage points in NR between 1 and 15°C thawing in favor of the colder temperature. Dunn et al. (199) observed no difference in NR figures between semen thawed at 40°C and semen thawed at 5°C. In contrast, a limited field trial (198) showed that fast thawing at 40°C and slow thawing at 5°C were both better than thawing at an intermediate temperature of 20°C. The differences were not statistically significant.

Although there is some disagreement on practical methods for thawing, it must be concluded from these data on rates of thawing for ampules containing 1 milliliter of semen, that thawing in water at 40°C or in ice water are the only methods that should be considered. The difficulty of keeping water at 40°C in the field, and the possibility of later cold shock if the ampules are held more than a few seconds at that temperature, eliminate 40°C. Thus thawing in ice water becomes the method of choice.

Current recommendations are to place at least a pint of water in an insulated container constructed so that ampules cannot freeze to the ice cubes or to each other (see Figure 17-18). If ampules freeze together or to ice cubes the rate of thawing could be altered. A typical thawing curve is presented in Figure 17-19. Although it is not known whether a critical temperature range analogous to that during freezing affects thawing survival, it is at this point that the rate of rewarming begins to change (see Figure 17-19). Thus thawing procedure could be critical.

Eight to 10 minutes should be allowed for the ampule to thaw and it should not be disturbed during that time. This recommendation differs from that of Boyd and Hafs (200). They compared motility and fertility of spermatozoa in ampules from which the "ice coat" was removed (fast thawing) to that of ampules allowed to thaw undisturbed in ice water (slow thawing). There were no statistically significant differences in motility or fertility between the methods. The results of two field trials, however, favored the slow thawing technique by 0.6 and 2.0 percentage points in NR, respectively.

From the results presented in Figure 17-20, it is recommended that the semen be used as soon as possible after thawing; if it is not used within one hour it should be discarded. This point is not universally agreed upon, however (201).

Thawing in Straws. The principles involved in thawing semen stored in straws should be similar to those that apply to semen stored in ampules. However,

the heat transfer characteristics of a long, slender plastic container require different thawing techniques. Aamdal and Andersen (159) used a staining technique to evaluate the results of thawing semen in straws. Thawing in water at 4°C, or in water or air at 20°C, was inferior to thawing in water at 35°C; and thawing at 35°C for 30 seconds was inferior to thawing at 75°C for 12 seconds.

French investigators (141, 148, 189) who were responsible for many of the techniques employed with the straw, thaw them in water at 35°C. In contrast, Canadian workers (155) thaw the 0.5-milliliter straw by "rubbing them back

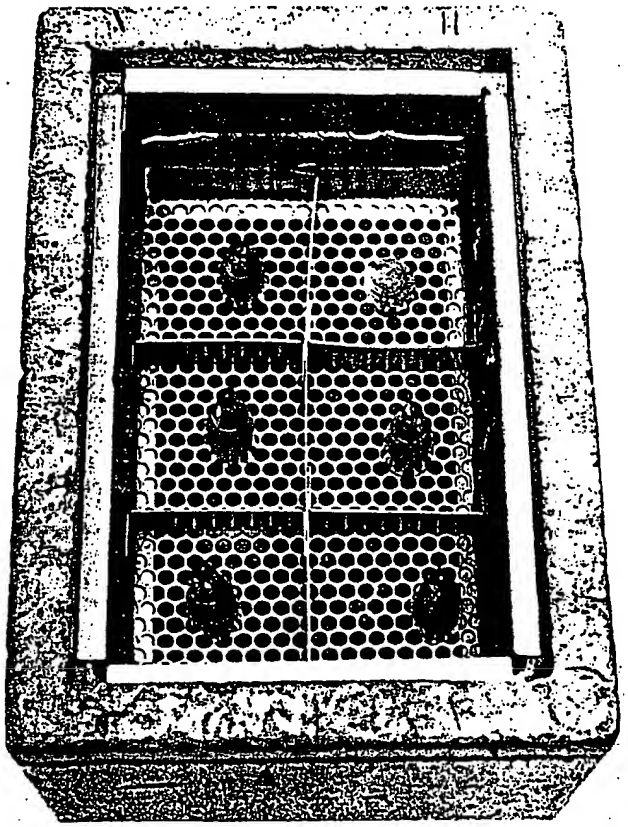

Figure 17-18. A thawing container designed to prevent ampules from freezing together or to ice cubes.

and forth between the palms of the hands." In a study by Bean (149) 16,000 to 21,000 first service inseminations per treatment were performed with semen in 0.5-milliliter straws thawed in ice water, warm water (30 to 35°C), or air. The 60–90-day NR rates were 71.1, 72.7, and 72.8 percent for thawing in ice water, warm water, and air, respectively. No statistical analyses were reported, but air thawing by wrapping the straw in a paper towel and placing it in the inside pocket of a technician's coveralls was adopted.

Robbins et al. (202) thawed semen in straws by plunging them into water baths maintained at temperatures of 5, 20, 35, or 75°C. In general, the more rapid thawing rates resulted in better motility and retention of the acrosomal cap (249). These results (202) disagree with the fertility results obtained by Bean (149). Although the ultimate test of a procedure is fertility (141), the results obtained by Robbins et al. (202) conform to the theoretical expectation—the faster semen is frozen the faster it should be thawed.

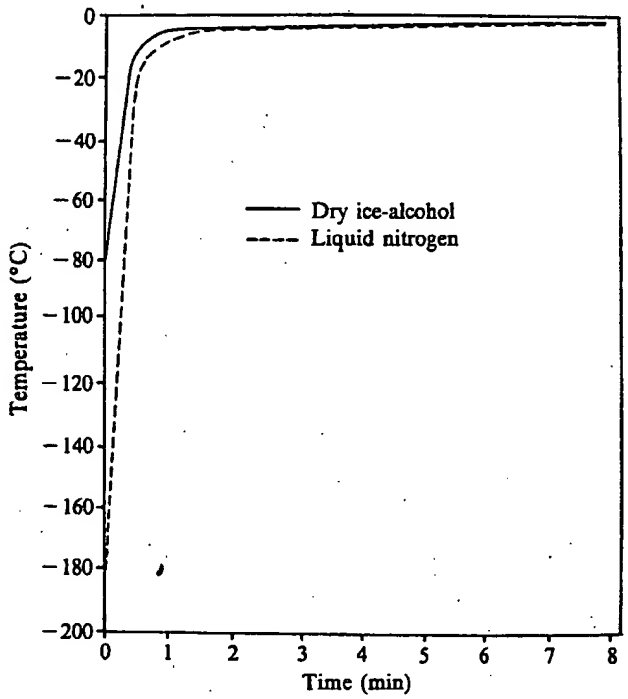

Figure 17-19. Typical thawing curve from −79° and −196°C for 1.0-milliliter glass ampules of bovine semen in ice water. Thawing rate measured by copper-constantan thermocouple located in the center of the ampule. [From Pickett. *A.I. Digest* 19(2):8-23. 1971.]

17-6 VARIATION IN SEMEN QUALITY

The individual bulls producing the semen are a major source of variation in all frozen semen experiments. Among the factors that can contribute to this effect are genetics, age, health, frequency of collection, and degree of sexual preparation (213, 218). Dairy and beef bulls vary in the quality of semen they produce and in the processing techniques needed for optimum fertility (219). Within types the heritability estimates of fertility in bulls vary (203, 204). However, the genetic variance of male fertility is relatively small and the selection pressure necessary to improve inherent breeding efficiency would be very high and could be made at the expense of other economically important traits (205). The major emphasis in commercial AI should therefore probably be directed to methods of obtaining and maintaining semen of greatest possible quality from available animals.

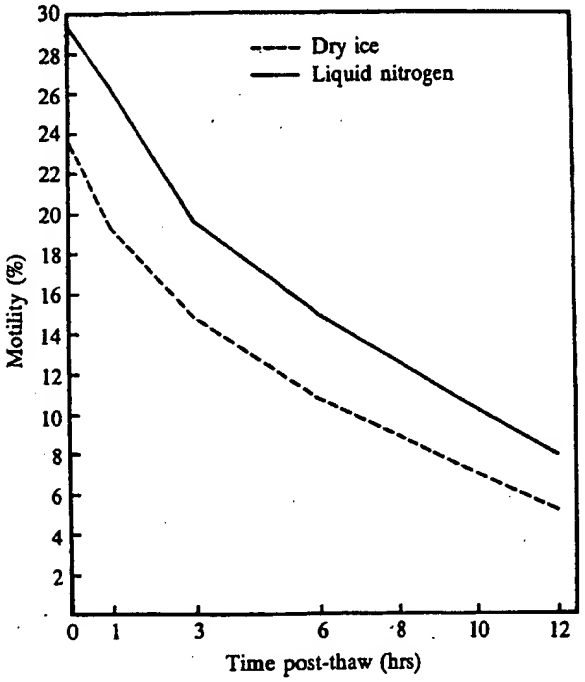

Figure 17-20. The effect of storage time at 5°C after thawing on percent motility of bovine spermatozoa. [From Pickett. *A.I. Digest* 19(2):8-23. 1971.]

The quality and fertility of semen produced declines as a bull ages (206, 207). Theoretically, semen can be collected from bulls at the height of their fertility and then frozen and stored for subsequent use. However, economic considerations and the delays inherent in evaluating and adopting innovations in semen processing and handling may preclude such policies (208).

The ability of semen to be frozen successfully, as indicated by post-thaw motility of several successive ejaculates, is often higher in the second ejaculate than in the first (210) and increases up to the fourth (209), especially if the semen has not been collected frequently on a regular schedule. For collection after 21 to 30 days of sexual rest the fertility of several consecutive ejaculates were not significantly different statistically, though the means favored the second (211). Short intervals between collection periods tend to eliminate the differences in freezability between two consecutive ejaculates (125). Proper sexual preparation has the same effect (212). The differences among ejaculates of the same bull are due to differences in the age distribution of individual sperm cells within ejaculates; this in turn affects the mean chemical composition (71, 72, 214, 215, 217) and resistance to cold shock (216). It is likely that some bulls are by-passed for AI because their semen freezes poorly under routine procedures that could be improved by generating special processing, freezing, and thawing procedures.

The number of cows to be bred each month of the year influences the amount of semen to be collected, processed, and frozen. The breeding of beef cows is confined to a relatively short season, usually in early spring or summer, depending on weather conditions and latitude. Dairy cows are bred more regularly throughout the year. Bulls proven to be genetically exceptional will undoubtedly be collected as frequently as possible, regardless of season. However, young bulls and bulls from which only a limited number of semen units are required should be collected during the time of the year when the semen is most fertile.

Salisbury (183) has presented evidence that at approximately 39° to 40° north latitude the observed seasonal variation in fertility is due primarily to an effect on the bulls of the seasonal differences in climate. The semen used for this study was frozen and stored at −79° to −88°C. The fertility of semen collected from May through October was significantly lower than that of semen collected during the other 6 months (see Table 17-10). These observations of decreased fertility in the summer months have been corroborated (191). In addition, Sullivan and Elliott (220) have found that semen collected in the fall and winter from bulls housed in a standard temperature-controlled environment and stored at −196°C was significantly higher in fertility (76.3 vs 74.2 percent, 60-90-day NR) than semen collected in the spring and summer (see Figure 17-21).

Custom freezing of semen is highly seasonal. Too often an owner will not present a bull for freezing of the semen until shortly before it is needed. This

is not good. Several ejaculates often must be collected before semen is obtained that is suitable for freezing. Waiting until the semen is needed could thus result in costly delays in the breeding schedule or in having to use a bull of less genetic potential.

From the results of these studies (183, 191, 220) it is recommended, economics and genetics notwithstanding, that semen should be collected for freezing during the fall and winter.

Table 17-10. The decrease in fertility (mean difference in 167-day nonreturns) between 1 and 2 months' storage and > 6 months' storage at about −79°C for the months in the year of insemination (cow response) and of semen collection (bull response).[a]

	Cow response	*Bull response*
Month	*Decrease after > 6 mo. storage*	*Decrease after > 6 mo. storage*
	(%)	(%)
January	13.8	7.0
February	13.8	11.8
March	16.6	8.4
April	13.5	11.5
May	12.8	16.8[b]
June	13.2	16.7[b]
July	11.3	20.9[b]
August	14.4	14.2[b]
September	7.3	21.4[b]
October	13.1	15.7[b]
November	14.6	12.8
December	14.7	8.9
Total insem.	174,307	176,432
Mean decrease	13.3	13.3

[a]From Salisbury. 6th Inter. Congr. Anim. Reprod. and Artif. Insem. Paris. II:1189–1204. 1968 (183).

[b]$P = 0.01$ that difference from mean is not due to chance.

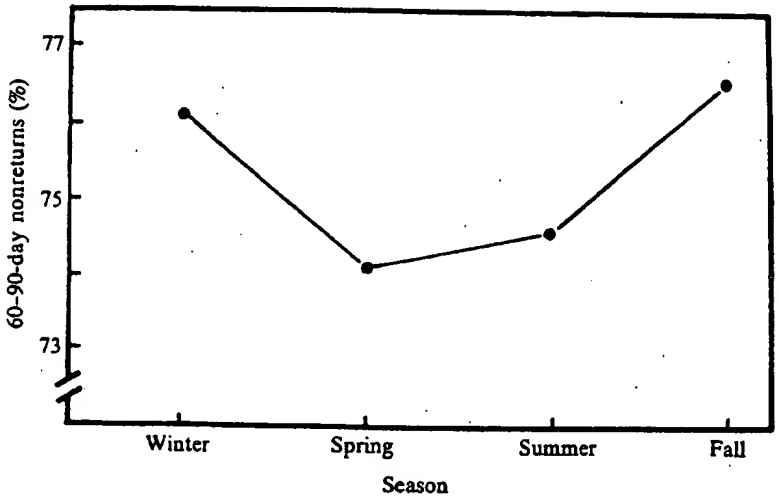

Figure 17-21. The effect of season on 60- to 90-day % NR [From Sullivan and Elliott. 6th Inter. Congr. Animal Reprod. and Artif. Insem. Paris. I:329-332. 1968.]

17-7 FREEZE-DRYING

The long term storage and subsequent shipment of spermatozoa at −196°C is costly and often inconvenient. If semen could be dried, placed in small packages, and stored at room temperature and still retain its fertility monumental savings could be realized. Attempts to recover motile spermatozoa following freeze-drying have met with variable success (9, 221–227, 238, 250). Meryman and Kafig (221) performed a single successful insemination with freeze-dried bull sperm, but they were unable to repeat this success (222). More recently, Graham et al. (250) have reported another successful insemination with freeze-dried sperm. It is believed that earlier attempts to freeze-dry semen failed because of the presence of glycerol in the extending medium. It is also true that the studies reporting successful recovery of motility upon reconstitution have been under conditions in which the semen samples have not been taken to a steady state of dryness (0.5 to 2.5 percent moisture) but have a higher moisture content. Very little success has been reported after the freeze-dried semen has been stored for a while; this may be related to the fact that when sperm cells are taken to a steady state of dryness motility cannot be recovered upon reconstitution with water. Reliable procedures for preserving bull spermatozoa by freeze-drying are not currently available, and much time and research are still necessary before the procedure will be of practical use.

The basic principles and techniques set forth in this chapter are intended to provide guidelines for obtaining maximum reproductive efficiency in a frozen semen program. Recommendations have been made according to available research data. While these techniques should provide optimal results under most circumstances, an awareness of the special needs of spermatozoa from individual bulls, and the flexibility required to meet these needs, should accompany every AI program.

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